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THE UNIVERSITY OF ALBERTA

Studies on Microbial Control of Mosquitoes in Central Alberta with Emphasis on the Hyphomycete *Tolypocladium cylindrosporum*

by

Mark Stanislaw Goettel

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

Department of Entomology

EDMONTON, ALBERTA

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COAL CREEK STUMP FARM

Site 31, Comp. 4, RR 1
Fanny Bay, B.C. V0R 1W0
Phone (604) 335-2263

21 October, 1986

Mark Goettel
Department of Entomology
255 Earth Sciences Building
University of Alberta, Edmonton

Dear Mark:

I would be pleased to have you include in your thesis our jointly published paper "Studies on the mosquito pathogenic hyphomycete Culicinomyces clavisporus", Goettel, Sigler and Carmichael, Mycoloigia 76:614-625,1984.

Sincerely,

J.W. Carmichael
Professor Emeritus.

University of Alberta — Inter-departmental Correspondence

to: Graduate Studies

date: October 30, 1986

our file:

from: Lynne Sigler, Research Associate
Devonian Botanic Garden
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Re: Thesis of Mark S. Goettel

I hereby authorize the inclusion of our joint paper entitled "Studies on the mosquito pathogenic hyphomycete Culicinomyces clavisporus" as a chapter in the thesis of Mark S. Goettel (Entomology).

Lynne Sigler

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PERMANENT ADDRESS:

214 Burke St.
Cowansville, Que.
J2K 2K1

DATED 19 January 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Studies on Microbial Control of Mosquitoes in Central Alberta with Emphasis on the Hyphomycete *Tolypocladium cylindrosporum* submitted by Mark Stanislaw Goettel in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisor

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External Examiner

Date...16 January, 1987

Abstract

Studies were conducted to determine the role of naturally occurring pathogens and parasites on larval mosquito populations in central Alberta and to evalute the potential of the Hyphomycete *Tolypocladium cylindrosporum* as a microbial control agent of mosquitoes. Naturally occurring pathogens and parasites generally had little effect on host populations. *Culicinomyces clavisporus* and *Smittium* sp. are recorded for the first time in Canada. The Canadian isolate of *C. clavisporus* was compared with isolates from the U.S. and Australia with regard to growth rate and colonial morphology and pigmentation. The Canadian and Australian isolates were more similar to each other than to the American isolate. *Tolypocladium cylindrosporum* is a relatively slow-acting pathogen with relatively low virulence to mosquitoes; massive doses are required to elicit a response. LC_{50}'s were in the order of 10^4 - 10^8 conidia/ml; LT_{50}'s were between 3 and 14 days. There was no increased pathogenicity after passage of the fungus 18 times through mosquito larvae. *Tolypocladium cylindrosporum* was easily propagated on a cellophane surface and wheat bran. The half-life of conidia stored at -20°C was 12.8 months. Mass applications of conidia in the field failed to induce an epizootic; however, infections were apparent in larvae that were transferred to laboratory conditions up to 29 days post application. The host-pathogen relationships between *T. cylindrosporum* and *Aedes aegypti* are complex. Principle

sites of invasion are through the base of the mandibles and maxillae and at the anus. Indications were that larvae are most susceptible immediately prior to molting; however, little fungal colonization of the hemocoel occurred at this time. Conidia ingested by larvae were excreted viable. The complexities associated with bioassay, strain selection, and field evaluation of *T. cylindrosporum* are discussed.

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I would like to express my appreciation to my supervisor, Dr D.A. Craig, and to the members of my advisory committee, Drs R. Currah and B.K. Mitchell for their guidance and criticism. Drs J.W. Carmichael, R.H. Gooding, M.A. Pickard, and J.R. Spence also served on my advisory committee at some time.

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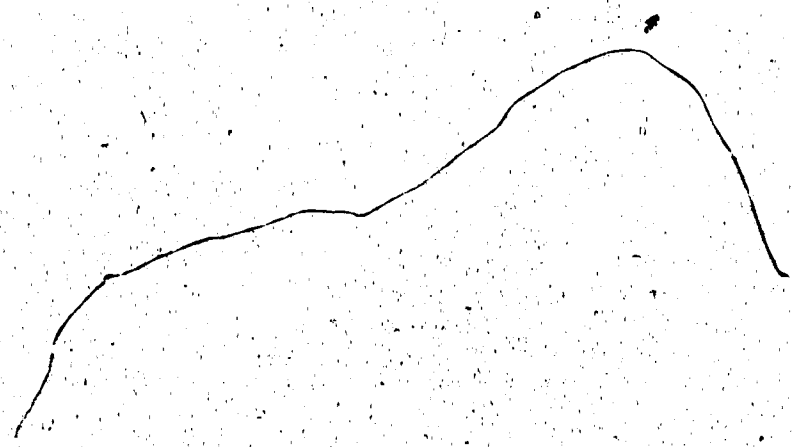

1. General introduction[1]

The blood-sucking behavior of mosquitoes has made them pests of enormous magnitude. Mosquitoes are vectors of disease-causing organisms such as malarial parasites, filarial worms, and arboviruses which kill or debilitate millions of people yearly. Heavy seasonal emergence of mosquitoes can significantly affect man's outdoor activities. Such outbreaks can also seriously reduce weight gain and milk production in cattle, sometimes causing death due to blood loss. For these reasons, mosquito control is a necessity in many parts of the world.

Since the mid 1940's we have relied mainly on synthetic chemical pesticides to control mosquitoes. Indeed, the mass application of chemicals such as DDT was initially so successful that it was once thought that diseases such as malaria could be eradicated. However, mass applications of these pesticides soon selected for resistant strains of mosquitoes, which made pesticide application virtually useless in many parts of the world. At the same time there developed a greater public awareness and concern over the deleterious long-term side-effects of pesticides, resulting in public pressure to discontinue their use. In addition, the cost of these chemical pesticides has increased markedly since the late sixties, making costs unbearable for developing countries.

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[1]A version of this chapter has been published. M. S. Goettel, 1985. Agriculture & Forestry Bulletin 8: 41-44.

The shortcomings of chemical pesticides have stimulated renewed interest in microbial control; the use of entomopathogens to regulate insect populations.

There are two basic control strategies for using biological control agents: (i)inoculation and (ii)inundation. The inoculative approach is the introduction of an exotic control agent so that it become established and provides adequate control. This approach is usually successful when employed against an introduced species that has become a pest, since too few, or no natural enemies exist in the area of its introduction. The inundative approach is application of large numbers of the agent into areas where it does not occur or where it occurs in levels insufficient to provide adequate control. In microbial control, this second approach is essentially the replacement of a chemical pesticide with a microbial one and entails repeated applications of the pathogen.

Since mosquitoes and most of their pathogens occur worldwide, the strategy that is presently considered as the most promising for microbial control of mosquitoes is inundation. It seems that the inoculative approach will be successful only with genetically altered pathogens. However, since genetic engineering is still in its infancy, microbial insecticides must be made available for mass applications in the same way as pesticides. In order for this to be accomplished, problems related to efficacy, safety, ease of application, economical mass production, storage, and

persistence must first be addressed.

1.1 Goals of the present research

The research was carried out under an Alberta Environment contract entitled "Investigations into Candidate Biological Agents for Mosquito Control in Alberta." The intent of the contract was to evaluate the potential of selected biocontrol agents as feasible and practical methods of mosquito control in Alberta.

The following specific goals are addressed in this study:

1) Evaluate the role of naturally occurring pathogens and parasites on mosquito populations in central Alberta.

2) Isolate pathogens occurring in the study area.

3) Develop mass culture methods for entomopathogenic hyphomycete fungi.

4) Evaluate storage of the hyphomycete *Tolypocladium cylindrosporum* at -20°C.

5) Increase pathogenicity of *T. cylindrosporum* by serial *in vivo* passage.

6) Develop a bioassay for *T. cylindrosporum* in mosquitoes.

7) Evaluate *T. cylindrosporum* against mosquitoes under field conditions.

8) Study the pathogenesis of *T. cylindrosporum* in mosquitoes.

The field under study is in an area of much active research; therefore, it is important to make results available to the scientific community without delay. For this reason, the thesis is presented in a "paper format." This allowed for publication of the studies as they were completed.

2. Field incidence of mosquito pathogens and parasites in central Alberta[1]

2.1 INTRODUCTION

Recently there has been much interest in use of pathogens and parasites as control agents of mosquitoes. Many potential control agents have been identified (see Jenkins 1964, Roberts and Castillo 1980, Roberts and Strand 1977, Roberts *et al.* 1983), but most of these represent limited collections and very little is known about their epizootiology. Very few studies have been made to determine fluctuations in parasite activity or the long term incidence of parasites. Such studies are required in order to better evaluate pathogens and parasites as potential biological control agents of mosquitoes. The present study was made to determine importance and seasonal incidence of naturally occurring mosquito pathogens and parasites in 10 selected pools from one location in central Alberta.

2.2 MATERIALS AND METHODS

2.2.1 Larval monitoring and diagnosis

Ten pools and ponds 4 km NW of Devon, Alberta (114°47'W, 53°23'N) were regularly monitored between June 1982 and September 1984. In 1982, monitoring began 15 June;

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[1]A version of this chapter has been submitted for publication. M.S. Goettel. J. American Mosquito Control Association.

in 1983 on 28 April; and in 1984 on 6 May. Five to 120 (most commonly 10 - 30) samples were taken from each pool on a weekly basis using a 350 ml capacity dipper. During each field trip, pool parameters such as water temperature (taken 10 cm below the water surface), pH, and conductivity were monitored. Dippers and the collectors' boots were rinsed in 5% household bleach after collection at each site in order to minimize the possiblity of spreading pathogens between study sites.

Field-collected immatures (i.e. larvae and pupae) were brought back to the laboratory, visually examined for presence of pathogens or parasites, counted, and placed into Bates' medium S (McLintock 1952) in trays or 500 ml plastic containers. Immatures were reared at 20°C until emergence or death. Dead larvae were removed daily, identified to species using the key of Wood *et al.* (1979), and then examined microscopically for signs of pathogens and parasites. At times, dead immatures were stored at 4°C for 24 - 48 hrs prior to examination.

2.2.2 Pathogen/parasite identifications/isolations

Pupae parasitized by mites were removed and placed in a separate container until adult emergence. Adults were then examined for mite infestation. Mites preserved in 70% alcohol were sent to Dr I.M. Smith, Biosystematics Research Centre, Ottawa for identification.

Microsporidian-infected immatures were kept at 4°C in sterile water and sent to Dr A. Undeen, Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida, for identification.

Whole cadavers with *Coelomomyces* infections were mounted on slides in lactofuchsin and the fungus was identified using the key of Couch and Bland (1985). Immatures infected with fungi other than *Coelomomyces* were bathed for 5 min in 50 µg/ml chloromycetin before being placed on the surface of Sabouraud dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin. Hyphal growth on the agar was subcultured until pure cultures were obtained. Saprolegniaceous fungi were subcultured onto 2.5% V-8® juice with 2% agar and sent for identification to Dr D.J.S. Barr, Biosystematics Research Centre, Ottawa.

Initial attempts were made to isolate pure cultures of most fungal pathogens, however, due to the high incidence of fungi in the order Saprolegniales and difficulties associated in their isolation and identification, this was discontinued after the first few months. Subsequently, only *Culicinomyces clavisporus* Couch, Romney and Rao was isolated; details are presented in Chapter 3. No attempts were made to isolate trichomycete fungi. These were preserved in lactofuchsin on slides and were sent for identification to Dr R.W. Lichtwardt, Department of Botany, The University of Kansas.

Peritrichida were not sent for identification. No attempts were made to identify or isolate possible bacterial pathogens.

2.2.3 Percent incidence estimates

Large numbers of mosquitoes were unaccounted for during rearing under laboratory conditions. Only dead immatures were examined microscopically for pathogens and parasites. As a result, 3 methods of estimating infection rates of field-collected mosquitoes were used:

(1) Minimum estimated % incidence= number infected/total collected x 100.

In this calculation, it is assumed that none of the missing immatures were infected and therefore it estimates the absolute minimum infection rate possible. This calculation was used for *Coelomomyces*, *Culicinomyces*, and Microsporidia. For Acari only the number of mosquito pupae were used in this calculation.

(2) Maximum estimated % incidence= estimated number infected/total collected x 100.

The estimated number infected was determined by multiplying the proportion of accounted dead immatures with infection by the total number collected, less the number of adults emerged. In this calculation, it is assumed that the same proportion of missing immatures were infected as those that were accounted for. This calculation also takes into account the number of adults emerging. This method was used for

Coelomomyces, *Culicinomyces*, and Microsporidia.

(3) Estimated % incidence= number infected/ number dead x 100.

In this calculation, it is assumed that the same proportion of all immatures collected were infected as those that died and were accounted for. This calculation was used for Saprolegniales, *Smittium* and Peritrichida.

2.2.4 Habitats

Details of the ten study sites are summarized in Table 2.1. Sites A, B, G, and I were not monitored in 1982. Sites C and D were dry in 1984 as a result of drainage operations nearby. All other sites were monitored for the entire study period as long as they contained water. Properties of the water were generally similar at all sites. Temperatures of between 3 and 5°C were recorded in early spring. Highest temperature was 25°C and was recorded at sites H and I. The highest temperatures at the other sites ranged between 20 and 24°C. Conductivity ranged between 105 and 1710 µmhos/cm and pH between 6.0 and 9.2. In 1982 and 1983, the total rainfall between June and August was approximately 30 cm while in 1984 during the same period it was 5.5 cm (Environment Canada, Edmonton International Airport 14 km SE of the study area). As a result, in 1984 most pools were dry by late summer.

2.3 RESULTS AND DISCUSSION

2.3.1 Mosquitoes

In general, pools were first colonized by Spring *Aedes* spp. Specimens collected included *Aedes cataphylla* Dyar, *Ae. euedes* Howard, Dyar, and Knab, *Ae. excrucians* (Walker), *Ae. fitchii* (Felt and Young), *Ae. flavescens* (Müller), *Ae. mercurator* Dyar, *Ae. pionips* Dyar, *Ae. punctor* (Kirby), and *Ae. riparius* Dyar and Knab. In early June, pools were generally colonized by *Culiseta alaskaensis* (Ludlow) followed later in the summer by *Cs. inornata* (Williston), *Cs. minnesotae* Barr, *Cs. morsitans* (Theobald), and *Culex territans* Walker. *Aedes vexans* (Meigen) colonized sites A, I, and J in early to mid-summer. Details are presented in Appendix 1.

In the present study identifications were attempted with specimens that had died and were generally in the process of deterioration. Because 4th-instar specimens in excellent condition are required for proper identification of Spring *Aedes* spp., the identifications are tenuous. Therefore, no attempts are made at establishing new host records for these Spring *Aedes* spp.

Many field-collected immatures disappeared while held in the laboratory. This occurred mainly when earlier instars were collected. Of 37,462 immatures collected in 339 collections, 45% were unaccounted for from the time of collection to the time the last individual either died or

emerged as an adult. Presumably this was a result of rapid decomposition and cannibalism of immatures held in the laboratory. Predators were also accidently introduced with the sample in some cases. Of the 55% of immatures that were accounted for, 67% (13,742) died and were examined microscopically for pathogens and parasites.

2.3.2 Pathogens and Parasites

Acari, fungi, Microsporidia, and Peritrichida were found associated with mosquitoes (Table 2.2). The most prevalent were saprolegniaceous fungi followed by commensal Peritrichida and Trichomycetes, and then microsporidian pathogens. Ectocommensal Peritrichida had the highest incidence. The least prevalent organisms were Acari, *Coelomomyces*, and *Culicinomyces*.

Incidences of pathogens and parasites were generally too low to make any comparisons between pools and years. There were no indications of correlations between pool parameters and infection rates. Details of pool parameters are therefore presented only for *C. clavisporus* as very little is known about its occurrence in nature.

2.3.3 Fungi

2.3.3.1 *Coelomomyces*

Only 4 larvae were found infected with *Coelomomyces* on 3 occasions and from two sites. All were identified as *Coelomomyces psorophorae* var *psorophorae* Couch with *Ae.*

vexans as the only host. The first infection was detected from site J on 23 July 1982. This pool was flooded as a result of heavy rain in the first week of July and was colonized by large numbers of *Ae. vexans*. The sample consisted of 11 dips which yielded 581 larvae (15 second, 7 third, 559 fourth-instar) and 43 pupae. The infected 4th instar larva died 3 days post collection.

The second and third occurrences were detected on 28 June and 12 July 1983 from site A. On 28 June, 44 larvae (3 first, 35 second, 6 third-instar) were collected in 10 dips and on 12 July, 33 larvae (8 first, 11 second, 14 third) and 50 pupae were collected in 5 dips. The single infected larva from the 28 June collection died 17 days post-collection while the 2 larvae from the 12 July collection died 6 and 9 days post-collection.

Coelomomyces psorophorae var *psorophorae* has been collected from many mosquito hosts and habitats world-wide (Couch and Bland 1985). In the United States it causes epizootics with infection levels of up to 96% in *Psorophora howardii* Coq. and *Cs. inornata* (see reviews by Chapman 1985, Lucarrotti *et al*. 1985). However, in a 6-year study of *Ae. cantans* (Meigen) in southern England, Service (1977) estimated an incidence level of less than 1 %.

In Canada *C. psorophorae* var *psorophorae* is widely distributed and well established in southern Alberta occurring in larvae of *Cs. inornata* and less commonly in *Ae. vexans* (Shemanchuk 1959, Zebold *et al*. 1979). Incidences of

up to 80% have been observed in *Cs. inornata* (Shemanchuk 1977) while in 1956, 12% of all *Cs. inornata* were infected (Shemanchuk 1959). *Coelomomyces* has also been reported from *Ae. trivittatus* (Coq.) in Manitoba (Taylor *et al.* 1980). These authors reported incidence rates of up to 56% in field collected mosquitoes, however, infections were apparent in blood fed adults only. Infection rates in the present study may therefore have been higher since adults were not examined. In the Manitoba study however, there was no evidence of infection in any blood fed *Ae. vexans* that were collected from the same pools where infected *Ae. trivittatus* were found. In related studies, adults of *Ae. sticticus* (Meigen) were found infected (R. Brust pers. commun. to Taylor *et al.* 1980). There are only 2 other records of *Coelomomyces* occurring in Canada; *C. borealis* var *giganteus* Couch and Bellamy collected in Ontario in *Ae. fitchii* (Felt and Young) and *Ae. stimulans* (Walker) and *C. canadensis* (Weiser and McCauley) Nolan collected from a chironomid larva in British Columbia (Couch and Bland 1985).

2.3.3.2 *Culicinomyces*

Culicinomyces clavisporus Couch, Romney and Rao was detected in every study year. The first infections occurred in collections of 12 and 19 August 1982 at site H. Properties of the water on the two collection dates were: 12 August: 15°C, 8.1 pH, 410 µmhos/cm conductivity; 19 August: 17°C, 7.8 pH, 412 µmhos/cm conductivity. The two samples consisted of 30 dips each. On 12 August, 215 larvae (40

first, 44 second, 70 third, and 61 fourth-instar) and 5 pupae were collected. Of 55 larvae identified, 64% were *Cs. minnesotae*, 32% were *Cx. territans*, and 4% were *Cs. inornata*. Of 170 larvae (12 first, 102 second, 24 third, and 32 fourth-instar) and 11 pupae collected on 19 August, 71 were identified; 63% were *Cs. inornata* and the remainder consisted of approximately equal numbers of *Cx. territans* and *Cs. minnesotae*. Four larvae of *Cs. inornata* were infected from the 12 August sample and 10 from the 19 August sample. Infected larvae died 8 - 13 days post-collection. A pure culture was obtained and has been deposited at the University of Alberta Microfungus Collection and Herbarium as UAMH 4618 (see chapter 3).

The second occurrence of *C. clavisporus* was found in the collection of 26 July 1983 from site G. Properties of the water were 17°C, 8.3 pH, and 550 µmhos/cm conductivity. Thirty dips yielded 69 larvae (50 first, 14 second, 4 third, and 1 fourth-instar). Of 45 larvae identified, 51% were *Cs. inornata* and the remainder were *Cx. territans*. A total of 17 infected larvae of *Cs. inornata* died between 7 and 22 days post-collection. A single infected larva of *Cx. territans* was found 23 days post-collection. A pure culture was obtained from this site and has been deposited at the University of Alberta Microfungus Collection and Herbarium as UAMH 4854.

The third occurrence of *C. clavisporus* was found in the collection of 12 June 1984 from site A. Properties of the

water were 12°C, 7.3 pH, and 600 µmhos/cm conductivity. Thirty dips yielded 259 second instar larvae of *Ae. vexans*. Two infected larvae died 5 days post collection. On the following weekly collections, only 1 larva and 4 pupae were collected from this site before it dried on 3 July for the rest of the study period.

Culicinomyces clavisporus was first isolated in 1972 as a contaminant of laboratory colonies of *Anopheles hilli* Woodhill and Lee in Sydney, Australia (Sweeney *et al.* 1973) and *An. quadrimaculatus* Say in Chapel Hill, North Carolina (Couch *et al.* 1974). Presumably, the fungus was introduced with water used to rear the larvae. Since these discoveries, considerable progress has been made in its evaluation as a potential biological control agent for mosquitoes (see reviews by Sweeney 1981a, 1985), however, little is known of its geographic range and occurrence in nature.

The first field isolation of *C. clavisporus* was made from larvae of *Ae. rupestris* Dobrotworsky collected from small rock pools in the partially dried bed of McCarrs Creek, Sydney, Australia (Russell *et al.* 1978). Further isolations have been made again from *Ae. rupestris*, and also from *Ae. rubrithorax* (Macquart), *Aedes* sp. as well as ceratopogonid and chironomid larvae collected in small rockpools in the Nattai River near Mittagong, N.S.W. (100 km south of Sydney) (Frances *et al.* 1985). More recently it has been recorded from larvae of *Cs. inconspicua* Lee breeding in a small groundpool at Mallacoota, Victoria (Frances 1986).

The recovery of *C. clavisporus* in Canada from a permanent pond, a marsh, and a semipermanent pool broadens the range of its known aquatic habitats (rockpools, streams, ponds, and lakes) and its geographic distribution (USA, Australia, Canada). *Culiseta inornata* and *Ae. vexans* are new records for mosquito hosts infected in nature; since the single infected *Cx. territans* larva died 23 days post collection, this was probably a laboratory acquired infection. This species was previously reported susceptible in laboratory challenge tests (Couch *et al.* 1974). The discovery of *C. clavisporus* in Canada provided an opportunity for comparative study of isolates from the three geographical regions (see chapter 3).

2.3.3.3 Saprolegniales

Fungi in the order Saprolegniales were the most prevalent, occurring in 50% of all collections. Incidences of up to 95% of all dead larvae were recorded and infections were noted in virtually all species collected. Initial identifications included *Saprolegnia ferax* (Gruith) Thuret from Spring *Aedes, Cs. alaskaensis*, and *Ae. vexans* and *S. hypogyna* (Pringshein) deBary from larvae of *Cs. alaskaensis* and from an unidentified mosquito pupa. Other occurrences noted as Saprolegniales may have included species in the genera *Achlya* and *Aphanomyces*; however, isolations of these species were not made.

Fungi in the order Saprolegniales are ubiquitous in distribution occurring in most collections of fresh water

(Liu and Volz 1976). Most are saprobes, although, a few species are important parasites causing disease in fish, fish eggs, frog eggs (Liu and Volz 1976) and *Daphnia* (Seymour *et al.* 1984).

Saprolegnia spp. have been isolated from mosquito larvae on many occasions (see Kalvish and Kukharchuk 1974, Roberts 1977); however, it is generally believed that these are infections of weakened or dying larvae. Even though laboratory studies have demonstrated high mortalities (i.e. Rioux and Achard 1956), little is known of the potential of *Saprolegnia* spp. as biocontrol agents of mosquitoes.

Recently a species of saprolegniaceous fungus, *Leptolegnia chapmanii* Seymour has been isolated on several occasions from mosquitoes in the USA (see Roberts and Panter 1985) and has been shown to be highly pathogenic to mosquitoes under laboratory conditions (McInnis and Zattau 1982).

This is the first report of *Saprolegnia ferax* in mosquitoes. *Culiseta alaskaensis* is a new host record for *S. hypogyna*. Previously it was reported from *Ae. excrucians* in the USSR (Kalvish and Kukharchuk 1974).

2.3.3.4 Trichomycetes

Many larvae were infected with the ectozootic *Amoebidium parasiticum* Cienk, however, since there is no reported evidence that its presence is detrimental to mosquitoes, details of its prevalence were not recorded. Many dead larvae were also infected with a *Smittium* sp.

Examination of slide-mounted larvae of *Ae. vexans* and *Cs. inornata* revealed that the fungus was probably *Smittium culisetae* Lichtwardt, however, Dr Lichtwardt noted "there is some question about the type of branching and the way the spores are borne on the fertile tips." I therefore refer to this fungus as *Smittium* sp. Larvae were found infected in up to 70% of the collections (i.e at site J in 1982) with up to 49% of dead larvae infected.

Trichomycetes are widely distributed and live obligately within the digestive tracts of arthropods with the exception of the Amoebidiales which live externally (Lichtwardt 1976). Trichomycetes of the genus *Smittium* were not generally thought to be detrimental to their hosts and there is some evidence that they may even be beneficial by providing certain nutrients (Starr *et al.* 1979). On the other hand, high mortalities resulted after first-instar larvae of *Ae. aegypti* (L.) were fed large numbers of *S. culisetae* spores (Williams and Lichtwardt 1972). It was speculated that this was a result of poor nutrition of the host. Recently, however, Sweeney (1981b) isolated *S. morbosum* Sweeney which was responsible for mortality rates of 50 - 90% in laboratory colonies of *An. hilli*. *Smittium morbosum*, unlike other species in this genus, was found to penetrate the midgut cells and at times the cells of Malpighian tubules. Others also report mortalities in mosquitoes as a result of *Smittium* infections (Coluzzi 1966, Dubitskii 1978 as cited by Sweeney 1981b).

In the present study, diagnoses of *Smittium* sp. were made only from dead larvae. It is therefore not known what proportion of the population was infected or if the fungus was a factor contributing to mortality. Diagnoses were most often made by observing a sporulating thallus protruding from the anus of the cadaver. This was also observed by Sweeney (1981b) in cadavers infected with *S. culisetae*. Since in the present study no dissections were made, the estimated incidence rate is probably conservative. There was no evidence of infections by *S. morbosum* (i.e. blackened appearance of invasion sites along the midgut).

In the present study *Smittium* sp. occurred in virtually all mosquito species collected with most observations in *Ae. vexans* followed by *Cs. inornata* (Table 2.3). This is the first report of a *Smittium* sp. from mosquitoes in Canada; *Cs. alaskaensis*, *Cs. minnesotae*, *Cs. morsitans*, and *Cx. territans* are new host records; it has also not been previously reported from any of the Spring *Aedes* spp. that occur in the Edmonton area. It has been previously recorded from *Ae. vexans* and *Cs. inornata* in Nebraska (Williams and Nagel 1980).

The only previous study conducted on the field incidence of *Smittium* spp. in mosquitoes was a 2 year survey in Nebraska by Williams and Nagel (1980). They found *Smittium* occurring most frequently in *Cs. inornata* with an annual infection rate of up to 53%; only low infection rates occurred in *Ae. vexans* (4%). The present results further

support their observations that *Smittium* does not appear to be host specific. Williams and Nagel (Loc. cit.) also speculated that *Smittium* was dependant upon the continued presence of hosts to maintain a population. The results of the present study indicate otherwise. *Smittium* sp. was commonly found in *Ae. vexans* and occurred in temporary pools shortly after they were flooded and had previously been dry for extended periods.

2.3.4 Microspora

2.3.4.1 Microsporidia

Microsporidians occurred at 8 of the 10 study sites. Prevalence was highest in 1982; but in 1984, only a single specimen was found infected (collected on 1 May from site I). Highest estimated incidence was 19 - 30%; overall incidence over the three years was under 2%.

There were difficulties in identification of microsporidians since diagnoses were made only after larvae had died. Most microsporidians were identified as *Amblyospora* spp. possibly *A. inimica* (Kellen and Wills) Hazard and *A. opacita* (Kudo) Hazard. Since it was not possible to obtain identifications for the majority of the infected specimens, I refer to these as Microsporidia.

Microsporidian parasites occur world-wide, infecting well over 100 mosquito species (Castillo 1980, Daoust 1983a, Hazard and Chapman 1977). Microsporidia of the genus *Amblyospora* have dimorphic development whose life cycle has

only recently been fully elucidated (Sweeney *et al.* 1985). The life cycle requires a copepod intermediate host; mosquitoes acquire the microsporidium from an infected copepod and it is then transmitted transovarially to the progeny. The microsporidian then forms uninucleate spores in the larva which dies in the fourth-instar. It was at this stage that the disease was diagnosed in the present study.

Field infections of microsporidia are usually 1% or less, although epizootics of 80 to 99% incidence are known (Andreadis 1983, Chapman 1974, Chapman *et al.* 1969).

Species found infected in the present study are presented in Table 2.3. New hosts records for Microsporidia include *Cs. alaskaensis*, *Cs. minnesotae*, and *Cs. morsitans*. Previous records include *Ae. vexans*, *Cs. inornata*, *Cx. territans*, and many *Aedes* spp. that occur in the Edmonton area in the spring; *Parathelohania* sp. occurring in *An. earlei* Vargas and *Nosema* sp. occurring in *Ae. excrucians* in Quebec and *A. khaliulini* Hazard occurring in in *Ae. communis* (DeGeer) at Churchill, Manitoba are the only previous Canadian records (Castillo 1980, Daoust 1983a, Hazard and Chapman 1977).

2.3.5 Ciliophora

2.3.5.1 Peritrichida

Peritrichs were abundant in most collections with up to 100% of dead larvae being infested and they occurred on virtually all species collected. The species involved was

probably a *Vorticella* sp.

Peritrichida occur frequently as epibionts on mosquito larvae and are usually considered not detrimental to their "host". There are, however, numerous reports of detrimental effects, including apparent mortality rates of up to 100% (see Clark 1977, Jenkins 1964). Canadian records of peritrichs on mosquitoes include Manitoba, Ontario, and Quebec (see Welch 1960 and references therein).

2.3.6 Acari

Acari or mites were found only in 1982 occurring from 5 out of the 6 sites monitored that year. The first occurrence of mites was on 24 June from site E at which time a single Spring *Aedes* sp. pupa was collected with 156 mites attached. These immatures were identified as belonging to the genus *Arrenurus* by Dr I.M. Smith. Mites encountered subsequently were not identified. The prevalence for 1982 was 11% with an estimated incidence rate of 0.5% (expressed as number of pupae infected/ total number of pupae collected).

Mosquito-parasitizing mites have a wide geographical distribution (Mullen 1975). *Arrenurus* spp. are the most common. Larvae attach to mosquito pupae and then transfer onto the adult during emergence and begin engorgement. When the mosquito returns to the water to oviposit, mites detach and continue their life cycle in the water. It is generally believed that mites reduce fecundity and longevity of their host and therefore have biological control potential (Smith

1983). Larvae of *Arrenurus* spp. are common in the tropics and subtropics and in some populations the prevalence of parasitization can reach 80%. In temperate regions mosquitoes are generally parasitized only occasionally, but prevalence within a population may be high (see references in Smith 1983).

In the present study, mites were found infecting Spring *Aedes* spp., *Cs. inornata*, *Cs. minnesotae*, and *Cx. territans*; *Cs. minnesotae* is a new host record. Previously mites were found on Spring *Aedes* and *Cs. inornata* in Alberta and on *Cx. territans* in Quebec (Leprince 1981, Mullen 1975).

2.3.7 Pathogens not found

It is interesting to note that neither viruses nor nematodes were found. There are no records of viruses from mosquitoes in Canada, although they are probably distributed worldwide (Federici 1985). Incidences of virus disease in nature are usually less than 1%, but epizootics with infection rates of up to 70% occur (Federici 1974, 1985). It is highly unlikely that in the present study infections of iridoviruses were overlooked. This disease is one of the easiest to diagnose as infected larvae are iridescent. Several suspect specimens were sent to Dr B.A. Federici, University of California, Riverside and were confirmed as being virus-free.

There are numerous records of nematodes from mosquitoes in Canada (Daoust 1983b, Petersen 1977, 1980) and most

infections are also easily diagnosed. Infection rates of up to 90% have been recorded (see Chapman 1974). It can be concluded that viruses and nematodes were either totally absent from mosquitoes at the 10 sites during the study period or occurred at such low levels that they were not detected.

2.3.8 Effects on host population

It is difficult to assess the field incidence of pathogens and parasites and their impact on the mosquito population especially when they occur at very low levels. Specific difficulties encountered in the present study include the following: (1) Transfer of field collected immatures to laboratory conditions probably stresses the individuals as evidenced by the high mortality rates witnessed. Stressed individuals may become targets for such "pathogens" as *Saprolegnia*. Most incidences of Saprolegniales were probably the result of such attacks; experiments with controls are required before the potential of these fungi as control agents is ruled out. (2) Since immatures were held in the laboratory, infections may have been acquired in the laboratory. As discussed above, this probably occurred with Saprolegniales since many cadavers were observed while the fungus was releasing zoospores. Some *C. clavisporus* infections were also probably laboratory-acquired as larvae died of infection up to 23 days post collection. (3) Large numbers of immatures

disappeared between the time of collection and the time the last individual either died or emerged as an adult.

In order to estimate incidence in the field as accurately as possible taking the above problems into consideration, 3 methods were used. Methods 1 and 2 were used for *Coelomomyces*, *Culicinomyces*, and Microsporidia to give an estimated range of % incidence. It is assumed that the possibility of laboratory acquired infections is minimal (in the case of *Coelomomyces* and *Culicinomyces*, the numbers found were so low that laboratory acquired infections would have little effect on the estimates in any case). Method 3 was used for *Smittium* sp. and Peritrichida as only dead larvae were diagnosed. Since these organisms are generally considered not detrimental to their "host", it can be assumed that the same proportion of all immatures collected were infected as those that died and were accounted for. If on the other hand, these organisms contributed to the death of the mosquitoes, this is an inaccurate method for estimating field incidence.

2.3.9 Concluding remarks

Even though difficulties were encountered in accurately estimating incidence in the field, pathogens and parasites appear to have had little effect on the larval mosquito populations studied. Similar observations were made by Service (1977) in England. In a 6-year study of *Ae. cantans*, he estimated that, although 95% larval and pupal mortality

occurred, few were killed by pathogens and parasites.

The many new locality and host records reported in the present study further demonstrate that the known geographical distributions of pathogens and parasites are only a reflection of the geographic distribution of entomologists interested in pathogens and parasites of mosquitoes (Chapman 1974, Platzer 1981). The extremely low prevalence and incidence of some pathogens demonstrates how difficult it is to establish such records unless long-term studies are undertaken. In terms of control of mosquitoes, the wide geographical distribution and low incidence of pathogens and parasites in nature indicates that the inoculative method of biological control may not be successful. Therefore, inundative use of these pathogens and parasites will probably be required. Further, if parasites and pathogens are ever to be fully exploited for mosquito control, a much better understanding of biotic and abiotic conditions causing epizootics is necessary.

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Table 2.1. Descriptions of pools in central Alberta monitored for mosquito pathogens and parasites between June 1982 and September 1984

Site	Habitat description	Vegetation'	Size(m)
A	Shallow, semipermanent pool in partially wooded area.	*Carex* sp.(d) *Populus* spp. *Salix* sp.(s) *Typha latifolia*(s)	15x34
B	Shallow, temporary roadside ditch.	*Caltha* sp.(s) *Salix* sp.(s) *Typha latifolia*(s)	1x8
C	Deep, semipermanent roadside pool with seepage from adjacent lake.	*Lemna minor* *Salix* sp.(s) *Typha latifolia*(s)	25x50
D	Same as C.	Same as C.	30x40
E	Shallow, temporary roadside ditch.	*Carex* sp.(d) *Typha latifolia*(s)	2x25
F	Large shallow marsh fed by stream.	*Caltha* sp. *Carex* sp.(d) *Populus balsamifera*(s) *Salix* sp. *Typha latifolia*(s)	210x210
G	Large shallow marsh.	*Carex* sp.(d) *Salix* sp.(s) *Typha latifolia*(s)	600x750
H	Permanent pond.	*Carex* sp.(d) *Lemna minor*(d) *Salix* sp.(s)	25x50
I	Shallow, temporary roadside pool.	*Carex* sp.(d) *Salix* sp.(s) *Typha latifolia*(s)	18x25
J	Same as I.	Same as I.	30x50

'd=dominant, s=scattered

Footnotes for Table 2.2

[1]Percent of collections where mosquitoes were found.

[2]Maximum % incidence in field collected immatures reared under laboratory conditions; for *Coelomomyces*, *Culicinomyces*, and Microsporidia = # infected/total collected x 100 to # estimated infected/total collected x 100; for Saprolegniales, *Smittium* and Peritrichida = # infected/# dead x 100; for Acari % incidence = # infected/total # of pupae collected at the site for the year x 100; see text for details.

[3]Pooled data.

Table 2.2. Summary of mosquito pathogen and parasite prevalence and incidence in 10 study sites in central Alberta

Site/year	No. collected	Prevalence[1] (estimated % incidence)[2]						
		FUNGI				MICROSPORA	CILIOPHORA	ACARI
		Coelomomyces	*Culicinomyces*	Saprolegniales	*Smittium*	Microsporidia	Peritrichida	
A								
1983	874	20(2-5)	-	50(39)	30(12)	20(2-4)	60(74)	-
1984	348	-	9(1)	54(73)	-	-	54(86)	-
B								
1983	604	-	-	57(37)	43(6)	-	71(79)	-
1984	1002	-	-	83(66)	8(1)	-	83(79)	-
C								
1982	819	-	-	50(71)	12(1)	19(4)	6(1)	19(6)
1983	211	-	-	40(80)	-	-	13(64)	-
D								
1982	540	-	-	33(19)	-	5(1-5)	-	17(5)
1983	121	-	-	8(60)	-	-	17(69)	-
E								
1982	3569	-	-	56(45)	56(12)	36(12-31)	9(6)	9(2)
1983	1301	-	-	57(45)	29(24)	5(1-2)	43(69)	-
1984	129	-	-	75(42)	-	-	100(100)	-
F								
1982	878	-	-	19(9)	6(12)	12(10)	6(8)	12(9)
1983	2460	-	-	76(53)	48(27)	9(4-5)	81(77)	-
1984	573	-	-	64(83)	14(2)	-	71(79)	-
G								
1983	632	-	5(26-38)	61(33)	22(8)	-	67(95)	-
1984	584	-	-	53(95)	-	-	60(82)	-
H								
1982	961	-	10(6-16)	26(26)	16(25)	-	-	5(2)
1983	349	-	-	40(48)	-	13(2-3)	40(66)	-
1984	334	-	-	57(61)	14(4)	-	71(68)	-
I								
1983	2992	-	-	33(41)	33(13)	7(10)	40(70)	-
1984	2165	-	-	61(90)	23(3)	8(3)	78(88)	-
J								
1982	4438	10(0.2-0.5)	-	60(43)	70(49)	80(3-10)	-	-
1983	2832	-	-	61(79)	61(16)	23(19-30)	77(75)	-
1984	8746	-	-	67(20)	60(8)	-	93(83)	-
Total[3]	37462	0.9(0.01-0.02)	1.2(0.09-0.2)	50.1(15)	23.0(4)	10.9(0.6-1.4)	43.4(30)	3.2(0.3)

Table 2.3. Summary of *Smittium* sp. and Microsporidia occurring in 10 study sites in central Alberta between June 1982 and September 1984

Host	Number of specimens collected	
	Smittium sp.	Microsporidia
Spring *Aedes*	10	27
Ae. vexans	287	113
Cs. alaskaensis	46	3
Cs. inornata	93	16
Cs. minnesotae	4	8
Cs. morsitans	2	5
Cx. territans	9	16
unidentifiable	41	43
TOTAL	491	231

3. Studies on the mosquito pathogenic hyphomycete *Culicinomyces clavisporus*[1]

3.1 INTRODUCTION

In 1972, observations of a fungal pathogen causing high mortality in laboratory-reared mosquito larvae were made independently in Sydney, Australia, and Chapel Hill, North Carolina (Sweeney *et al.* 1973, Couch *et al.* 1974). The American isolate was named *Culicinomyces clavisporus* by Couch, Romney and Rao (1974) (as "*clavosporus*", see discussion under 3.3.1 Nomenclature). Sweeney and his co-workers in Australia considered their isolate to be congeneric (Sweeney 1975, Sweeney and Painter 1977), but only recently confirmed its identity as *C. clavisporus* (Sweeney *et al.* 1982). In their study, they compared the original isolate from Sydney with one from Chapel Hill and concluded that they were conspecific. Although they noted some differences between the strains, they were unable to evaluate these differences until more isolates could be studied.

Sweeney and his associates studied *C. clavisporus* as a potential biocontrol agent for mosquitoes. Its host range, mode of pathogenesis, efficacy in field trials, and potential pathogenicity to other insects and as well as other animals have been well documented (Sweeney 1981a, b,

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[1]A version of this chapter has been published. Goettel, M.S., L. Sigler, and J.W. Carmichael. 1984. Mycologia 76: 614-625.

1985). Its geographic range and occurrence in nature are less well known.

In 1982, I isolated *C. clavisporus* from field-collected larvae of *Culiseta inornata* (Williston) in Alberta, Canada (see chapter 2). Prior to this, the fungus was known only from two widely separated geographic regions. Infections in field collected mosquitoes have been known previously only in *Aedes rupestris* Dobrotworsky larvae (Russell *et al.* 1978, Frances, pers. comm.). *Culiseta inornata* is a new host record for this fungus.

The discovery of *C. clavisporus* from Canada coincided with an announcement (Anon. 1982b) that an American and an Australian isolate were available from the American Type Culture Collection (ATCC). This provided an opportunity for a comparative study of isolates from the three geographic regions. From this comparison, I concluded that the three strains are conspecific and that the form-genus *Culicinomyces* should be maintained for now.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of the Canadian strain

Between 15 June and 6 October 1982, approximately 12 ground pools and ponds in the Devon, Alberta, area (114°47'W, 53°23'N) were monitored for mosquito pathogens. Records of mosquito density, water pH, temperature, and conductivity were kept for each body of water. Larvae

collected weekly by dipping were placed in pans in the laboratory at 20°C and observed daily. Dead larvae were removed and examined microscopically for invasion by fungal hyphae. Infected larvae were placed in 200 ml Bates' medium S (McLintock 1952) containing 20 laboratory-reared larvae of *Cs. inornata*. Laboratory-reared larvae which became infected were bathed for 5 min in 0.12% sodium hypochlorite (2% household bleach), and then 5 min in 50 µg/ml chloromycetin before being placed on agar plates. Hyphal growth on the agar was subcultured until pure cultures were obtained, but it was difficult to obtain a culture free from bacterial contaminants.

A culture and dried specimens are maintained at the University of Alberta Microfungus Collection and Herbarium as UAMH 4618. Subcultures were deposited at the American Type Culture Collection (ATCC) (52635), the Commonwealth Mycological Institute (280342), the Centraalbureau voor Schimmelcultures (75583) and Insect Pathology Resource Center, Boyce Thompson Institute,(ARSEF) (964).

3.2.2 Other isolates

American and Australian isolates were obtained from the ATCC as 38490 (=UAMH 4658) and 46258 (=UAMH 4659), respectively. Four additional isolates from North Carolina were received from ARSEF. Their numbers are UAMH 4848 (ARSEF 372) mosquito, 4849 (ARSEF 582) *An. quadrimaculatus*, 4850 (ARSEF 584) *Anopheles quadrimaculatus*, all three from Chapel

Hill, and 4851 (ARSEF 706) mosquito, from North Carolina. One additional isolation from Alberta was made from *Cs. inornata* collected in July 1983, and numbered UAMH 4854 (see chapter 2). All of the last five isolates were obtained too late to be included in the comparative growth studies, but they were examined microscopically.

3.2.3 Growth studies

Media consisted of Pablum mixed cereal agar without chloromycetin (PCA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) containing 50 µg/ml chloromycetin, oatmeal-salts agar (OAT) (all recipes in Padhye *et al.* 1973), cornmeal agar with yeast and dextrose (CM+, Sweeney *et al.* 1982), and nutrient agar (NA) and broth (NB, Difco). Small fragments of growth (approximately 1 mm^3) from one-week-old cultures on OAT were transferred to Petri plates containing each of the media listed above. Each strain was inoculated to three replicate plates and the colony diameters were measured after 21 days. The cultures were incubated at 25°C and exposed to fluorescent ceiling lights on an irregular basis (usually 8-10 h/day, 5 days/wk).

One liter flasks containing 500 ml of NB were inoculated with conidia from each of the three strains. The cultures were incubated at 20°C and agitated at approximately 170 oscillations/min on a Burrell wrist action shaker. Observations on sporulation and development of

pellicle were made daily for 28 days.

3.2.4 Scanning electron microscopy

Techniques used for preparation of specimens were adapted from Brown and Brotzman (1979). Small blocks of agar with mycelial growth were removed from 47 day cultures on OAT and placed onto glass microscope slides. Each specimen was vapor-fixed for 2 days at room temperature in a sealed dish containing 4 ml of 2% osmium tetroxide in distilled water, then quick-frozen in liquid nitrogen and lyophilized overnight. Infected larvae were prepared by 2 methods: (1) vapor fixation as described above but air-dried for 6 days rather than lyophilized and, (2) fixation in 1% osmium tetroxide, 2% Kodak Photoflo in distilled water for 2 days at 4°C, dehydration through a graded ethanol series to 95% ethanol, followed by air drying for 4 days.

All dehydrated specimens were affixed to SEM stubs using conductive silver paint and then sputter-coated with gold in a Nanoteck-Semprep 2. Observations were made on a Cambridge S 250 SEM operated between 10-20 KV.

3.3 RESULTS AND DISCUSSION

3.3.1 Nomenclature

Originally, the epithet given to this species was "*clavosporus*." Recently, the orthographic variant "*clavisporus*" has been adopted by some authors (Onions 1979,

von Arx 1981, Hall 1982, Sweeney *et al.* 1983). In 1982, Hall claimed that the correct spelling is "*clavisporus*." However, roots of both Latin and Greek origin are combined, and there is some disagreement about the proper vowel to use in such cases. Classical mycologists used "*clavisporus*" for this combination (for example, see Saccardo 1886, p. 631), for this reason, I have adopted and recommend the usage of "*clavisporus*" rather than "*clavosporus*."

3.3.2 History of the American Type Culture Collection isolates

Couch *et al.* (1974) deposited the type specimen of *C. clavisporus* at the University of North Carolina Herbarium, but no mention was made of deposition of a living culture. Strain data for ATCC 38490 (Anon 1982a) is "J.N. Couch, mosquito, *Anopheles quadrimaculatus*." This strain was not deposited at ATCC until 1979 (Jong, pers. comm.). During the period from 1973 to 1979, several isolations of *C. clavisporus* have been made from infected larvae at Chapel Hill (Humber, pers. comm., Panter, pers. commun.). ATCC 38490 is presumably one of Couch's early isolates, but precise information on the source of some of the Chapel Hill isolates is lacking. It appears that the isolate on which the original description was based is no longer available (Humber, pers. comm.).

Strain data for ATCC 46258 (Anon 1983) is R.C. Russell S1 235, mosquito, *An. amictis hilli*. This strain was

deposited at ATCC January 18 1982 (Jong, pers. comm.). According to Panter (pers. comm.), this is the original Sydney isolate, and it is the strain used by Sweeney *et al.* (1982) in their comparative study of the Australian and American strains. In their publication, no strain numbers were given for either strain, but the American strain was one of Couch's isolates (Panter, pers. comm.).

3.3.3 Habitat and distribution

Culicinomyces clavisporus was first observed causing infection in laboratory reared mosquito larvae in two widely separated localities, Sydney, Australia, and Chapel Hill, N.C. Presumably, the fungus was introduced with the water used to rear the larvae. In the U.S. (Couch *et al.* 1974) the water came from University Lake, a man-made reservoir of several acres near Chapel Hill (Sweeney 1983). In Australia, the water originated from McCarr's creek, a small perennial stream near Sydney (Russell *et al.* 1978, Sweeney 1983).

Russell *et al.* (1978) provided the first report of an infection in field-collected mosquitoes; larvae of *Ae. rupestris* breeding in small rock pools in the partially dried bed of McCarr's creek. Further isolations have been made from *Ae. rupestris* occurring in rock pools of varying sizes at Nattai River near Mittagong (100 km south of Sydney) (Frances, pers. comm.).

Debenham and Russell (1977) demonstrated that an infection of *C. clavisporus* originating in larvae can be

carried into the adult stage. Infection of the adult mosquito may be important in dispersal of the fungus.

In Alberta, *C. clavisporus* was found on larvae of *Cs. inornata* occurring in a permanent pond north of Devon (Winterburn area). The pond measures approximately 25 x 50 m; it has a muddy bottom with a grassy periphery and was covered with duck weed (*Lemna* sp.) by mid-summer. Infection in *Cs. inornata* was detected only in larvae collected on 12 and 19 August 1982. Properties of the water on the two collecting dates were: 12 August 15°C, 8.1 pH, 410 µmhos/cm conductivity at 22°C; 19 August 17°C, 7.8 pH, 412 µmhos/cm conductivity at 22°C.

The two samples consisted of 30 dips taken from the pond periphery in the grassy area. Each dip yielded approximately 6-7 larvae/350 ml. On August 12, 215 larvae (40 first, 44 second, 70 third, and 61 fourth-instar) and 5 pupae were collected. Of 59 larvae identified, 59% were *Cs. morsitans* Barr, 30% were *Culex territans* Walker and 10% were *Cs. inornata*. Of 170 larvae (12 first, 102 second, 24 third, and 32 fourth instar) and 11 pupae collected on August 19, 71 were identified; 63% were *Cs. inornata* and the remainder consisted of approximately equal numbers of *Cx. territans* and *Cs. morsitans*.

From the collection of August 19, I observed a larva of *Cs. inornata* in which hyphae had penetrated the external cuticle to form conidiophores bearing terminal and lateral flask-shaped phialides (Figs. 3.1, 3.2). Obovate conidia

were produced from the tip of the phialide. The fungus was tentatively identified as *C. clavisporus* and later confirmed by comparison with the two isolates obtained from ATCC.

Only larvae of *Cs. inornata* were found to be infected with *C. clavisporus*, 4 from the August 12 sample and 10 from the August 19 sample. However, more larvae may have been infected than diagnosed since many dead larvae disintegrated or were devoured by other larvae. Conversely, infection in some of the field collected larvae may have occurred following exposure to other infected larvae in the laboratory, since the infected larvae did not die until 8-9 days after being collected. Consequently, the ratio of infected to collected larvae may not reflect the infection rate in the field.

Infection by *C. clavisporus* occurred when the population of *Cs. morsitans* and *Cx. territans* in the pond was being replaced by *Cs. inornata*. *Culiseta inornata* was present in the pond until 6 October 1982. Two more instances of *C. clavisporus* infections in mosquitoes in Alberta were found in subsequent years (see chapter 2).

The recovery of *C. clavisporus* from a permanent pond and a marsh broadens the range of its known aquatic habitats (rock pools, streams, ponds and lakes), and its geographic distribution (USA, Australia, Canada). *Culiseta inornata* is a new record for a mosquito host infected in nature; only *Ae. rupestris* has been previously reported.

3.3.4 Growth studies

The appearance of colonies of *C. clavisporus* growing on solid agar media has been described previously (on NA and CM+ by Sweeney *et al.* 1982; on malt agar and PDA by Onions 1979). We compared growth rates of the three isolates on six media (Table 3.1, Fig. 3.3). The U.S. strain grew slightly faster except on SDA (Fig. 3.3b). It also grew well on CM+ (Fig. 3.3c), the medium on which it has been routinely maintained since its isolation (Sweeney *et al.* 1982). In contrast, the Australian strain grew slowest on NA (Fig. 3.3d), the medium used to maintain the stock culture in Sydney (Sweeney *et al.* 1982).

The Canadian and Australian strains had similar growth rates but colonies of the latter were frequently glabrous and tough, sometimes splitting the agar after several weeks' growth (Fig. 3.3b). Colonies of the first Canadian isolate developed abundant aerial mycelium, but colonies of the new isolate, UAMH 4854, are more glabrous and aerial mycelium develops slowly. On some media (PDA, PCA), colonies of both the Australian and Canadian strains developed a darkly pigmented surface mycelium (Fig. 3.3a).

Culicinomyces clavisporus is unique among the entomopathogenic Hyphomycetes in its ability to produce conidia on larvae which are submerged (Roberts and Humber 1981, Roberts and Sweeney 1982). Other fungi produce conidia only when infected larvae float to the surface and the mycelium is exposed to air.

In liquid shaker culture (NB), the American strain produced more abundant hyphae. The mycelial growth of the American and Australian isolates appeared darkly pigmented by macroscopic observation, whereas, the mycelium of the Canadian strain was not pigmented. The Australian strain sporulated readily; the Canadian strain moderately, and the American strain sparsely under these conditions. A similar observation was reported by Roberts and Sweeney (1982) for the American and Australian isolates.

3.3.5 Conidium development

Conidiogenesis in *C. clavisporus* is characterized by the formation of terminal and lateral phialides which are borne either singly (Fig. 3.4), or in complex whorls at irregular intervals along the conidiophore (Figs. 3.5, 3.6, 3.8), or in penicillate structures which may be simple (Fig. 3.7) to complexly branched. Phialides are flask-shaped, 8-15 x 2-3 μm, tapering at the neck to a diameter of 0.5-1 μm (Figs. 3.5, 3.6) sometimes with a minute collarette. Occasionally, lateral phialides are more swollen at the base, tapering abruptly at the neck; these shorter phialides measure 8 μm or less (Fig. 3.10). The conidia are obovate, 5-7.5 x 1.5-3 μm, and they accumulate in slimy masses (Fig. 3.9).

We also noted two aspects of conidiogenesis which have not been previously illustrated. The first is the formation of conidia of a second type which are oval to cylindrical,

unicellular, 2-3 x 1-2 μm. These smaller conidia occur on phialides adjacent to ones producing the larger obovate conidia (Figs. 3.13, 3.14). They also accumulate in slimy masses.

In addition to simple phialides I observed polyphialides (Figs. 3.4, 3.11, 3.12) with two, or rarely more than two, openings. Inman and Bland (1983) also observed polyphialidic development.

3.3.6 Strain variation

In 1982, Sweeney *et al.* compared the growth and sporulation of an American and an Australian isolate in agar culture and on larvae, summarized the differences and concluded that they were conspecific.

Our study has confirmed the variability among three isolates of *C. clavisporus*. The strains vary in their colonial morphology and growth rates (Table 3.1, Fig. 3.3) and in their conidium development. Conidial dimorphism occurred in all three strains but the small conidia were more abundant in the American and Australian strains. Roberts and Sweeney (1982) reported globose rather than obovate conidia in some batches of conidia of the Australian strain grown in 750 liter fermenters. They stated that the round conidia were less virulent to mosquito larvae. It may be that the small "conidia" are spermatia. We saw the small conidia in agar slide culture using PCA as the medium, but not in submerged broth culture. Polyphialides occurred more

commonly in the American strain. The four additional isolates from North Carolina also developed conidia of both types, and polyphialides.

Neither I nor Sweeney *et al.* (1982) have found the penicillate, complexly branched structures which were produced by the original American isolate growing on *Ae. atropalpus epactius* Dyar (Couch *et al.* 1974: fig. 18). Sweeney *et al.* (1982) observed only sparse development of phialides which occurred singly or in whorls of 2 or 3.

3.3.7 Taxonomy of *Culicinomyces*[1]

Culicinomyces is a monospecific genus having taxonomic affinities to several other hyphomycete genera. The endoparasitic nematode-destroying fungus *Meria coniospora* Drechsler (1941) produces conidia similar in size (4-7 x 1.8-2.5 μm) and shape, but its conidia sometimes terminate in small bud-like protrusions (Fig. 3.16) which are adhesive structures. The conidia of *M. coniospora* are produced successively from small pegs (Figs. 3.15, 3.16, arrows) which develop laterally near the apex of each conidiogenous cell. These pegs appear to elongate during formation of successive conidia.

The size, shape, and arrangement of conidiogenous structures in *Beauveria* Vuill., *Tolypocladium* Gams, *Verticillium* Nees and *Paecilomyces* Bain. are similar to *Culicinomyces*. Each of these genera also contain

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[1]Section 3.3.7 contributed by L. Sigler.

entomopathogenic species. *Beauveria* differs in forming a sympodially proliferating conidiogenous axis. From each node on the geniculate rachis, only a single conidium is produced. In *Tolypocladium*, the conidiogenous structures are phialides which are borne singly or in false whorls on narrow, loosely branched conidiophores (Bissett 1983). Frequently, the phialides are grouped in dense clusters. The phialides are short and swollen basally, narrowing abruptly to a filiform neck which is frequently bent. In his comprehensive review, Bissett (1983) has enlarged *Tolypocladium* by the addition of several species. Two species formerly treated in *Verticillium* including *V. balanoides* (Drechl.) Dowsett *et al.* (1982) and *V. microsporum* Jaap are described as having subulate phialides arranged in verticils. By this treatment, the distinction between *Tolypocladium* and *Verticillium* becomes less clear, and these species are probably better left in *Verticillium*. Bissett separates the genera on the basis of the broader conidiophore main axis in *Verticillium*.

The concept of *Verticillium* was broadened by Gams (1971) in his Section *Prostrata* to include many species which display erect conidiophores, but a more recent treatment (Domsch *et al.* 1980) indicates that the genus is not well defined. We consider *Verticillium* to be characterized by aculeate phialides without collarettes. The phialides are divergently arranged in verticils and borne at intervals along the length of undifferentiated or

well-developed, erect, sometimes dematiaceous conidiophores. Conidia are borne in slime. Von Arx (1981) treated *Cullcinomyces* under *Verticillium*, but in *Cullcinomyces* the phialides are adpressed in whorls rather than divergently arranged.

Paecilomyces is similar to *Cullcinomyces* in the arrangement and shape of phialides, but in *Paecilomyces* the conidia are formed in true chains (Gams 1978, Subramanian 1979, Minter *et al*. 1983). *Cullcinomyces* is further differentiated from both *Paecilomyces* and *Verticillium* by its polyphialides.

Hirsutella Pat. species occur on arthropods and many have teleomorphs in the Clavicipitaceae. Polyphialides, which occur occasionally in *Cullcinomyces*, are more common in *Hirsutella*. Both mono- and polyphialidic conidiogenous cells are borne laterally or terminally in synnemata, but mononematous forms are also included (Minter and Brady 1980, Samson *et al*. 1980, Evans and Samson 1982a, b). The mononematous forms of *Hirsutella* can be differentiated from *Cullcinomyces* by the phialides which are usually borne singly, arising from undifferentiated hyphae more or less at right angles. None of the species develop the adpressed whorls of phialides characteristic of *Cullcinomyces*. The phialides are swollen basally and taper gradually or abruptly at the neck which may be twisted in a helical rotation. Furthermore, conidia are borne singly or in small groups, frequently in a characteristic mucous sheath which

may be pigmented. In the majority of mononematous species, the conidia are ellipsoidal or in the shape of an orange segment (*fide* Minter and Brady 1980). Further investigation may show that *C. clavisporus* could be accommodated in *Hirsutella*; I am reluctant to propose a transfer at this time.

Culicinomyces clavisporus differs from all other entomopathogenic species of Hyphomycetes in its aquatic rather than terrestrial habitat. It has been found in nature only as a pathogen of mosquito larvae and certain other aquatic Diptera. It is unique in its ability to produce and disperse conidia under water.

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Table 3.1. Mean colony diameters of three *Cullcinomyces clavisporus* isolates grown for 21 days at 25°C on six selected media

STRAIN	MEDIA					
	PDA	PCA	CM+	OAT	NA	SDA
AMERICAN (ATCC 38490)	3.8[1]	3.0	2.8	3.1	1.8	1.8
AUSTRALIAN (ATCC 46258)	1.9	1.6	1.6	1.9	1.0	2.1
CANADIAN (UAMH 4618)	2.3	1.7	1.6	1.9	1.5	1.8

[1] in cm

Figures 3.1 to 3.3. *Culicinomyces clavisporus*.

Figures 3.1 to 3.2. Hyphae emerging through cuticle of *Culiseta inornata* larva to form conidiophores bearing terminal and lateral flask-shaped phialides bearing obovate conidia. (UAMH 4618). (Bar=20μm)

Figure 3.1. Phase contrast.

Figure 3.2. Scanning Electron Micrograph.

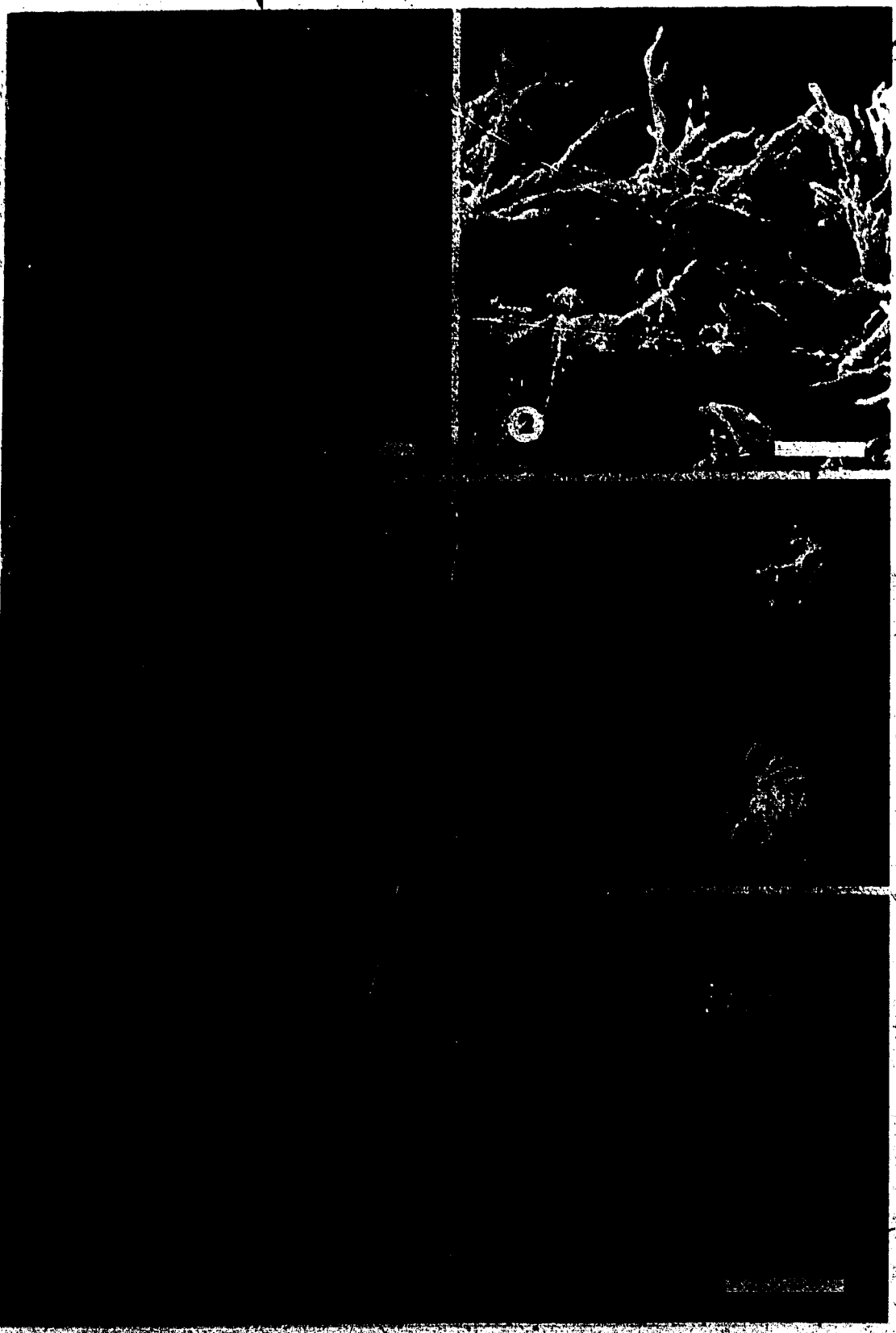
Figure 3.3. Colonies of three strains grown on different media for 21 days. American strain (UAMH 4658) at left; Canadian strain (UAMH 4618) at upper right; Australian strain (UAMH 4659) at lower right. (Bar=2cm)

a. on Potato dextrose agar.

b. on Sabourauds dextrose agar.

c. on Corn meal + yeast and dextrose agar.

d. on Nutrient agar.



igures 3.4 to 3.9. *Cullcinomyces clavisporus*

Figure 3.4. Simple phialide and polyphialide (arrow) borne singly. (UAMH 4658) (Bar=20μm)

Figures 3.5 to 3.6. Phialides borne in whorls at irregular intervals along the conidiophore. (UAMH 4618)

Figure 3.5. (Bar=20μm)

Figure 3.6. (Bar=5μm)

Figure 3.7. Penicillately branched phialides bearing obovate conidia in slimy masses. (UAMH 4618) (Bar=20μm)

Figure 3.8. Phialides borne in single whorl. (UAMH 4618) (Bar=2μm)

Figure 3.9. Obovate conidia covered in slime. (UAMH 4659) (Bar=4μm)

Figures 3.10 to 3.14. *Culicinomyces clavisporus*

Figure 3.10. Basally swollen, short phialides borne in whorl. (UAMH 4658) (Bar=2μm)

Figure 3.11. Simple phialides and polyphialides borne in whorls at intervals on conidiophore. (UAMH 4618) (Bar=5μm)

Figure 3.12. A polyphialide with two openings. (Bar=4μm) UAMH 4618.

Figures 3.13 to 3.14. Small, oval to cylindrical and larger obovate conidia borne in slimy masses from adjacent phialides. (Bar=20μm)

Figure 3.13. UAMH 4659.

Figure 3.14. UAMH 4618.

Figures 3.15 to 3.16. *Merla coniospora*. (UAMH 4730). Obovate conidia produced from small pegs (arrows) borne laterally near apex of each conidiogenous cell. Conidia are equipped with bud-like protrusions which are adhesive structures.

Figure 3.15. (Bar=20μm)

Figure 3.16. (Bar=100μm)

4. A simple method for mass culturing entomopathogenic hyphomycete fungi[1]

4.1 INTRODUCTION

In recent years there has been a resurgence of interest in use of fungi for control of insect pests. Before a fungus can be registered as a biological control agent, laboratory bioassays, field trials, as well as research on storage, viability, and formulation are required.

For laboratory bioassays, relatively little infectious material is needed and it is usually obtained by culturing the fungus in standard petri dishes on defined or semi-defined media. However, this procedure becomes too tedious and expensive for the production of larger quantities of infectious material usually required for subsequent studies.

Most techniques for mass culturing fungi have been developed towards harvesting fungal metabolites using submerged liquid fermentation; however, infectious propagules are required for biological control. Furthermore, most entomopathogenic Hyphomycetes, with the exception of *Culicinomyces clavisporus* Couch, Romney and Rao, do not sporulate readily in liquid culture (Roberts and Sweeney 1982). Most of these Hyphomycetes produce blastoconidia (also referred to as "blastospores" or "hyphal bodies") in submerged culture, but these have virtually been abandoned

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[1]A version of this chapter has been published. M.S. Goettel. 1984. J. Microbiological Methods 3: 15-20.

for use against insects due to their short-lived nature (Ferron 1981, Roberts and Humber 1981). Some progress has been made in obtaining phialidic conidia in submerged culture, but these are usually less virulent and have shorter half-lives than surface produced conidia (Roberts and Sweeney 1982). Consequently, most production methods for acquiring infectious material use a surface phase method incorporating either solid media, semi-solid media or still liquid culture (Hall and Papierok 1982, Roberts and Sweeney 1982). Still liquid culture requires specialized equipment (Kybal and Vlček 1976, Samšiňáková *et al*. (1981) and broths can be easily contaminated by bacteria or other fungi. Most surface phase methods require milling of the product, but heat build-up during the milling process can affect spore viability (Hall and Papierok 1982, Roberts and Sweeney 1982). Furthermore, the end product consists of a mixture of substrate, conidia, and mycelial fragments.

Recently Daoust and Roberts (1983) and Daoust *et al*. (1983) used a simple method for producing conidia of *Metarhizium anisopliae* (Metsch.) Sorok. on rice in heat resistant nylon oven bags or autoclavable high-density polyethylene bags. Conidia were harvested by drying the rice/fungus mixture in enamel pans in a laminar flow cabinet and then passing it through a sieve twice. Sieving is time consuming and exposes the fungus and worker to possible contamination. The drying step can also significantly reduce conidial viability in some species of fungi (Hall and

Papierok 1982).

The need for producing *Tolypocladium cylindrosporum* Gams conidia for use in small scale field trials against mosquitoes has prompted me to develop a method whereby large quantities of fungi, free from contaminants and substrate, can be produced cheaply and conveniently.

In 1944, Fleming and Smith suggested using cellophane to separate mold colonies from the agar substrate on which they were grown. This method has been adopted for routinely preserving mold colonies in a herbarium (Carmichael 1963). Consequently, by adopting the use of cellophane as a barrier between the fungus mycelium and nutrient source, large quantities of fungus free from substrate contamination can be obtained.

The culture method developed uses metal roasting pans, bran, cellophane, and autoclave bags. This paper demonstrates the success of this culture method with several entomopathogenic Hyphomycetes.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Inoculum

Cultures of *Tolypocladium cylindrosporum* (UAMH 4561), *Metarhizium anisopliae* (UAMH 421), *Beauveria bassiana* (Bals.) Vuill. (UAMH 4748), *Verticillium lecanii* (Zimm.) Viegas (UAMH 4842) and *Culicinomyces clavisporus* (UAMH 4618) were obtained from the University of Alberta Microfungus

Collection and Herbarium. These fungi were inoculated onto the surface of 50 ml of Pablum mixed cereal agar (Padye *et al.* 1973) in 200 ml culture flasks and were incubated for two weeks at 20°C. Conidial suspensions were obtained by adding 100 ml of sterile 0.5% Tween® in distilled water to the culture flasks and shaking. Conidial counts were made using an improved Neubauer hemocytometer and the required amounts of 0.5% Tween® were added to give final concentrations of 1 x 10^4 conidia/ml. These suspensions were then used as the inoculum.

4.2.2 Culture methods

Approximately 70 gm of wheat bran and 700 ml of distilled water were combined in tin cookware roasting pans (36 x 26 x 4 cm). The bran mixture was allowed to imbibe water for several minutes. Pre-cut sheets of cellophane[1] (40 x 32 cm) were soaked in distilled water for 15 minutes to remove the polyethylene glycol used as a softener in P24. A single sheet was then layered over the bran mixture leaving at least 2 cm of the cellophane extending up onto the sides of the pan. This prevented the inoculum from seeping below the cellophane surface into the bran. In placing the cellophane on the bran surface, care was taken to exclude all air bubbles. Each pan was then placed into a large autoclave bag (61 x 71 cm, Fisher Scientific Co.) and sealed by folding the bag under and over the pan (1.5 times) and

[1] P24, British Cellophane Ltd., Bath Road, Bridgewater, Somerset TA6 4PA, England.

taping it with two strips of masking tape (Fig. 4.1a). The pans were autoclaved for one hour at 138 kilopascals (20 psi, approx. 125°C) in a steam autoclave. Care was taken not to exceed this temperature as it is near the melting point of the bags. Due to the presence of aerobic spore bearing bacteria in the bran, lower temperatures or shorter autoclave times resulted in inadequate sterilization. Extreme care was taken in cooling the autoclave as slowly as possible to prevent the bran mixture from boiling, thereby, causing the cellophane to lift off the bran surface.

Following removal from the autoclave, the pans were allowed to cool to room temperature and water which condensed onto the surface of the bags during autoclaving was sponged off. A small area on the bag surface was swabbed with alcohol and 10 ml of the inoculum was injected onto the surface of the cellophane using a sterile hypodermic syringe. The puncture hole was sealed with a piece of masking tape and the pan was gently rocked so that the inoculum was evenly distributed over the entire surface of the cellophane. At least four replicate pans were prepared for each species. Initially the pans were incubated at 20°C with a photoperiod of L/D 17/7 for two weeks, but it was found that at this temperature and incubation time the yield of *C. clavisporus* was very low (Table 4.1) and further trials increasing both incubation time to three weeks and temperature to 25°C were performed with this species.

The pans were removed from the autoclave bags and the cellophane sheet with adhering fungus was gently lifted off the bran surface. Some pans were frozen for photographing at a later date (Figs. 4.1b-f).

4.2.3 Determination of Yield

The mycelial mat was scraped off the cellophane surface using a spatula and was placed in enameled trays and air dryed for 4-5 days. The dryed mixture of conidia and mycelia was then scraped out of the pans, powdered in a mortar and pestle and weighed. Conidial yields were determined by rehydrating 0.1 g of the powder in 10 ml of 0.5 % Tween® at 4°C for 24 hrs. The rehydrated mixture was suspended by mixing on a vortex for two minutes. The number of conidia in the suspension was determined using an improved Neubauer hemocytometer. Mean numbers of conidia per g, per pan, and per cm^2 were calculated.

4.3 RESULTS AND DISCUSSION

Within one year of continuous use, many of the roasting tins rusted to such an extent that perforations occurred. Subsequently, it is recommended that enamel, stainless steel, aluminium, or autoclavable polypropylene trays be adopted for long term use. I have used all of the above types of trays with no apparent differences in fungal growth. For routine inoculation of pans with *T. cylindrosporum*, a continuous dispensing pipet attachment

(Econopet®) is used. This labor saving device reduces the chance of contamination when large numbers of pans are being inoculated.

The autoclave bags can be reused several times. The conidia/mycelia can be easily scraped off the cellophane surface or the cellophane can be rolled up with the adhering fungal mat and stored frozen at -20°C for later use. The effects of this method of storage on conidial viability of *T. cylindrosporum* are presented in chapter 5.

The yields obtained using the tin pans are summarized in Table 4.1. Yields of fungi cultured on solid substrates can approach 10^{10} conidia/g dry weight (Hall and Papierok 1982). The yields obtained in the present study for all fungi except *C. clavisporus* were comparable. Sparse growth was obtained for *Culicinomyces* and *Metarhizium* (Figs. 4.1b, c), while the growth in the other three fungi was abundant (Figs. 4.1d-f). However, in this study, no attempts were made to optimise the conditions to obtain maximum conidial yield except for *C. clavisporus*. These conditions would vary for each fungal species and even each isolate.

An analysis of variance of the *C. clavisporus* results showed that there was a significant increase in dry biomass with increase of incubation time but not with increase in temperature. There were no significant differences in the conidial yield/g or per cm^2 with increased temperature or incubation time. These results indicate that 25°C might be above the optimum temperature for growth and sporulation of

this species. The mycelia were glabrous and tough with very little aerial growth (Fig. 4.1b) at both temperatures and incubation times.

This experiment has shown that using the culture method described, large quantities of contaminant-free conidia of various entomopathogenic Hyphomycetes can be obtained with a minimum of equipment and cost.

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Table 4.1. Mean conidial yields of five species of fungi using the cellophane-bran culture method.

SPECIES (Incubation length and temperature)	No. of pans	BIOMASS[1] Per pan (g dry wt.)	NUMBER OF CONIDIA[1] Per gram ($\times 10^{13}$)	Per pan ($\times 10^{13}$)	Per cm^2 ($\times 10^{9}$)
Tolypocladium cylindrosporum (14 days, 20°C)	6	5.2±0.38	3.4±0.37	17.3±1.5	1.3±0.15
Verticillium lecanii (14 days, 20°C)	4	8.9±1.01	2.8±0.30	20.8±3.14	2.3±0.35
Beauveria bassiana (14 days, 20°C)	4	5.3±0.36	1.5±0.38	7.5±2.15	0.80±0.235
Metarhizium anisopliae (14 days, 20°C)	6	4.0±0.55	0.61±0.097	2.3±0.81	0.08±0.033
Culicinomyces clavisporus (14 days, 20°C)	6	1.73±0.216	0.070±0.0040	0.117±0.0102	0.012±0.0011
Culicinomyces clavisporus (21 days, 20°C)	6	2.26±0.161	0.076±0.0131	0.165±0.0261	0.018±0.0035
Culicinomyces clavisporus (14 days, 25°C)	5	2.01±0.197	0.067±0.0215	0.14±0.047	0.015±0.0050
Culicinomyces clavisporus (21 days, 25°C)	7	2.76±0.170	0.076±0.0147	0.21±0.037	0.022±0.004

[1] ± 1 Standard Error.

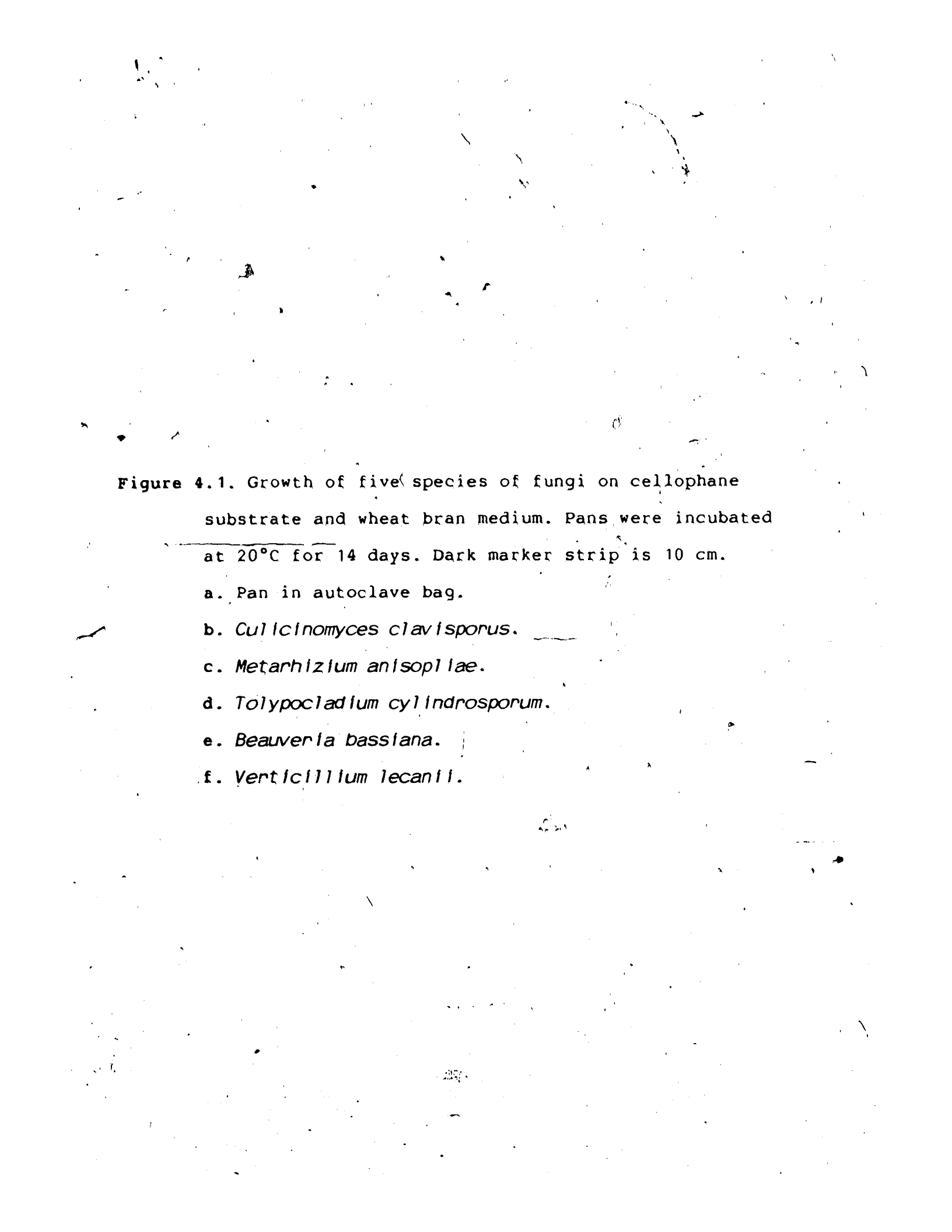


Figure 4.1. Growth of five species of fungi on cellophane substrate and wheat bran medium. Pans were incubated at 20°C for 14 days. Dark marker strip is 10 cm.

a. Pan in autoclave bag.

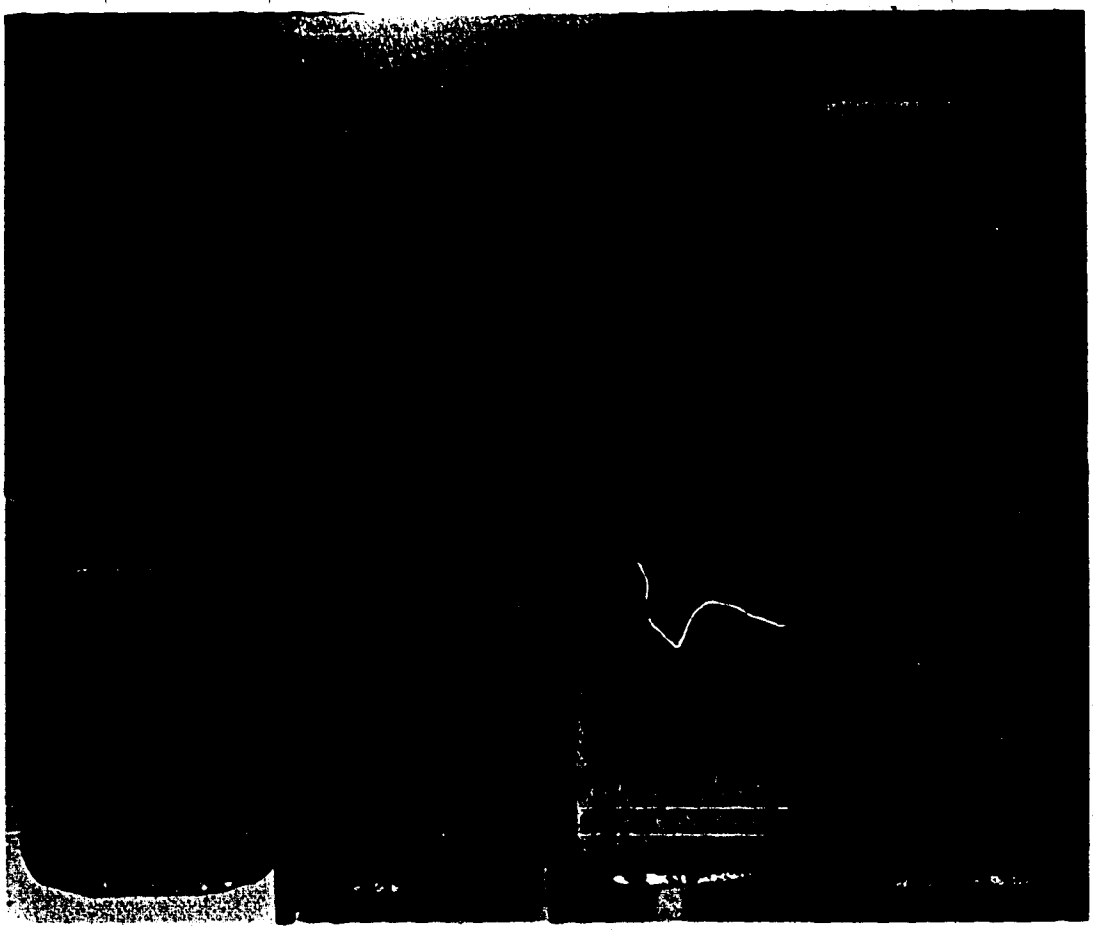
b. *Cullcinomyces clavisporus.*

c. *Metarhizium anisopliae.*

d. *Tolypocladium cylindrosporum.*

e. *Beauveria bassiana.*

f. *Verticillium lecanii.*



5. Conidial Viability of the Mosquito Pathogenic Hyphomycete *Tolypocladium cylindrosporum* following Prolonged Storage at -20° C[1]

5.1 INTRODUCTION

The need for obtaining relatively large amounts of *Tolypocladium cylindrosporum* Gams conidia for use in small-scale field evaluations against mosquitoes has necessitated development of methods for production and storage of this fungus. Preservation of fungal cultures at low temperatures is often successful (e.g. Carmichael 1962, Mazur 1968, Samson 1982). Muller-Kogler and Zimmermann (1980) reported that the best temperature for the storage of 7 species of entomopathogenic Hyphomycetes was -18°C. They demonstrated longevity for up to 9 years, but quantitative data on conidial viability was not recorded. On the other hand, *Culicinomyces clavisporus* Couch, Romney and Rao conidia lost considerable viability after only a few months at -20°C (Sweeney 1981). Storage was more successful at -70°C, although, only 50% of the conidia were viable following 6 months at this temperature.

A recently developed method for producing fungi on a cellophane surface and a wheat bran medium (chapter 4) was used to obtain large quantities of *T. cylindrosporum* conidia. The present chapter reports the effects of storage at -20°C on conidial viability.

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[1]A version of this chapter has been submitted for publication. M.S. Goettel. J. Invertebrate Pathology.

5.2 MATERIALS AND METHODS

A Californian isolate of *T. cylindrosporum*, INRA 3 (Institute National Recherche Agronomique, La Miniere, France)= UAMH 4561 (University of Alberta Microfungus Collection and Herbarium), was cultured for 2 weeks at 20°C according to the method described in chapter 4. Sheets of cellophane, with adhering fungal mat, were rolled up, placed in plastic bags, and stored at -20°C. One cm squares were randomly cut from different layers and sections of the frozen cellophane roll each month for up to 32 months and placed in 10 ml of sterile distilled water. Conidial suspensions were prepared by mixing on a vortex mixer for 2 min. Conidial counts were made using an improved Neubauer hemocytometer and appropriate dilutions were made to obtain suspensions of 1 x 10^4 conidia/ml.

Conidial viability was determined by spreading 0.1 ml of the suspension onto the surface of potato dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin in a standard Petri dish. Numbers of viable and non-viable conidia were counted on the plate surface under phase contrast at a magnification of 200X following incubation at 25°C for 24 hrs. Counts were made in 5 fields of view per plate examined. Each value for percentage viability is based on observations of between 263 and 2,961 spores, but most commonly 500 - 1500. Conidia were considered viable if they swelled to a "barbell" or "peanut" shape. This swelling occurs just prior to the formation of a

germ tube (see chapter 9).

5.3 RESULTS AND DISCUSSION

The conidial half-life was approximately 12.8 months(Fig. 5.1). In one instance, 79% viability was obtained after storage for 18 months at -20°C. Indications are that *T. cylindrosporum* does not suffer thermal shock (i.e. an initial large mortality as a result of freezing) and that viability decreases at a slow progressive rate. There was a linear relationship between conidial viability and time held at -20°C. Death was probably the result of a progressive dessication and subsequent increase in the concentration of solutes both within and outside of the cell (Mazur 1968).

Daoust and Roberts (1983) obtained a similar but more rapid progressive decline in conidial viability for a strain of *Metarhizium anisopliae* (Metsch.) Sorok. However, a different strain suffered a reduction from 93 to 56% viability in the first 2 weeks after freezing (i.e. possible thermal shock); thereafter, viabilities remained relatively stable (36-56%) for up to 24 months. They speculated that susceptibility to freezing differed according to strain. However, these differences in viability as well as those obtained in the present study with the one strain of *T. cylindrosporum* could be a result of differences in cooling rates; an important factor in the survival of conidia after freezing and thawing (Mazur 1968). Cooling rate would vary

according to such factors as the initial temperature of the fungus and its distance from the cooling coils in the freezer. In the present study the fungus was rolled in cellophane; therefore, the outside layers would freeze faster than the inner layers. Since squares of cellophane were randomly cut from different layers and sections of the cellophane roll this could account for the variability in the viability rates obtained.

Gardner and Pillai (1986) studied various methods of storage of *T. cylindrosporum*. The most successful method was storage of conidia in distilled water at 4°C. Using this method, they obtained viabilities of 86% after two years, however, results were based on one 10 ml sample. Since this method may be a good alternative to storage at -20°C, studies on the prolonged storage of large quantities of *T. cylindrosporum* conidia under these conditions are warranted.

I do not consider storage of *T. cylindrosporum* at -20°C as a commercially acceptable method. It is, however, a convenient method for accumulation of this fungus for experimental studies. Conidia stored at -20°C for approximately 2.5 years retained about 10% viability. With yields of approximately 10^8 conidia/cm^2 (see chapter 4), yields would still be approximately 10^7 viable conidia/cm^2 or 10^9/gm dry weight. The accumulation of the equivalent of 1 kg dry weight of *T. cylindrosporum* conidia using this method of storage has enabled preliminary field trials to evaluate this fungus for mosquito control (see chapter 8).

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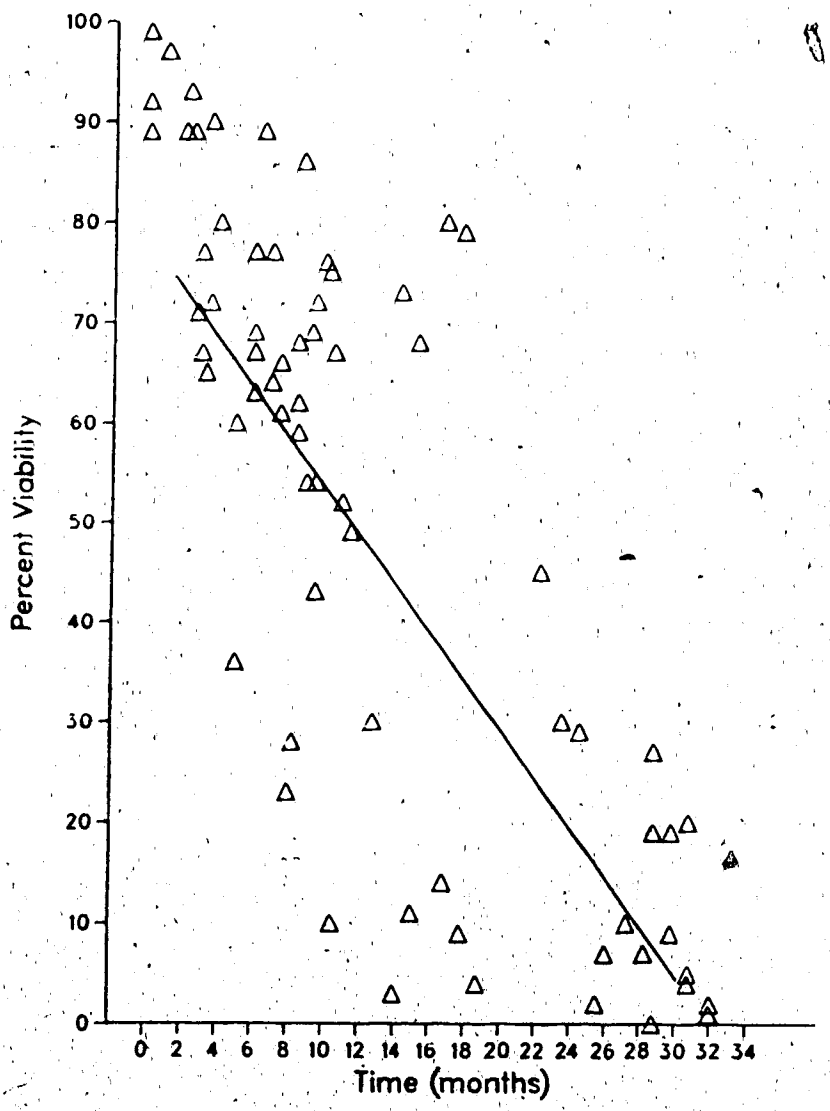
Figure 5.1. Viability of *Tolypocladium cylindrosporum* conidia stored at -20°C as a function of time. The regression equation is:

viability = 83.4 - 2.6 x time.

(r^2 = 0.66; $p<0.00001$).


Percent Viability
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Time (months)

6. Serial *In vivo* passage of the entomopathogenic hyphomycete *Tolypocladium cylindrosporum* in mosquitoes[1]

6.1 INTRODUCTION

Enhancement of virulence in entomogenous fungi is a particularly neglected area of research (Roberts and Yendol 1971). It is generally believed that virulence increases following successive passage through a host and, conversely, decreases following repeated subculturing on artificial media (see Aizawa 1971, Roberts and Yendol 1971). By using single-spore isolates (clones) of *Beauveria bassiana* (Bals.) Vuill., Samšiňáková and Kálalová (1983) demonstrated spontaneous mutants with increased virulence. Enhancement of virulence using parasitic selection pressures has been accomplished by passage of fungal strains through a host (Daoust and Roberts 1982; also see references in Ignoffo *et al.* 1982). Such changes are thought to be due to gradual selection of genotypes. This paper reports on serial passage of the mosquito pathogenic hyphomycete *Tolypocladium cylindrosporum* Gams through larvae of *Culiseta inornata* (Williston) and *Aedes aegypti* (L.) conducted in an attempt to select for a more pathogenic strain.

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[1]A version of this chapter has been submitted for publication. M.S. Goettel. Canadian Entomologist.

6.2 MATERIALS AND METHODS

A Californian isolate of *T. cylindrosporum* [TC3=INRA 3 (Institute National Recherche Agronomique, La Miniere, France)= UAMH 4561 (University of Alberta Microfungus Collection and Herbarium)] was used. Serial passage of isolate TC3 through mosquito hosts was performed 18 times. Passages 1 to 7 were through *Cs. inornata*. Due to difficulties encountered in rearing this species, subsequent passages were through larvae of *Ae. aegypti* (8-18). Three replicates of 20 2nd-instar larvae were each exposed to 200 ml of 1 x 10^3 and 10^4 conidia/ml solutions in Bates' medium S (McLintock 1952) at 20°C. The first dead larva with mycosis was bathed in a 50 µg/ml sterile solution of chloromycetin for 5 - 10 min and was placed onto the surface of potato dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin (PDA-SP) in a Petri dish. After one week of incubation at 20°C the fungus arising from the larva obtained from the 10^3 conidial suspension was subcultured onto PDA-SP and was used as the inoculum for the next serial passage. A culture was also stored at -20°C. On several occasions there was no mortality which could be attributed to the fungus (i.e. presence of mycelia in the hemocoel) at 10^3 conidia/ml. In such instances, isolations for subsequent passages were made from a dead larva exposed to the 10^4 conidia/ml suspension.

After 18 passages, 2 completely randomized assays were performed at 20°C. The isolates used in these assays were

the original (TC3), passages # 6, 13, and 18 which had been stored at -20°C. Each assay consisted of 3 replicates of 20 2nd-instar larvae exposed to 4 concentrations of conidia and a control according to the method outlined in chapter 7. Mortality was recorded daily and final mortalities were analyzed by the two-way analysis of variance.

6.3 RESULTS AND DISCUSSION

There was no evidence of increased pathogenicity after passage of the fungus 18 times through mosquito larvae (Table 6.1) ($F=0.44$, $p>0.1$). Selection for a more pathogenic strain should be most successful when the only genotypes isolated after each passage are those with increased virulence. The chances of this genotype selection would be increased in cases where only one spore, i.e. the most virulent mutant, successfully infects the host and replicates within it as a clone. However, this seldom occurs. In theory, with decreasing dosage, the probability of singly infected animals increases (Huber and Hughes 1984). It would also be expected that probabilities of single infections would also increase as the infection sites are limited. In the present study, successive passages were made at the lowest dose to increase the probability of singly infected larvae. However, genetic selection of pathogens which are ingested may pose unique difficulties. In such cases, it is conceivable that most animals that have succumbed to the pathogen will harbor infection propagules

in the gut that did not contribute to pathogenesis yet will germinate in the cadaver and produce propagules which will be used in the successive passage. In the present study, no apparent increased virulence was obtained by passaging *T. cylindrosporum* 18 times through mosquitoes. Likewise, Ignoffo *et al.* (1982) detected no increase of virulence after 12 successive *in vivo* passages of *Nomuraea rileyi* (Farlow) Samson through larvae of *Trichoplusia ni* (Hübner). Since in both of the studies larvae were inoculated by feeding, it is possible that there was no selection for genotypes that were involved in the infection process.

In contrast, Daoust and Roberts (1982) were successful in selecting for increased virulence in *M. anisopliae* after only one passage through a mosquito host. Since this fungus has a limited infection site, namely the perispiracular valves on the siphon, the chances of single infections are presumably increased. Therefore, the chances of reisolating non-infective or avirulent spores is also lessened. Selection of pathogenic genotypes of *T. cylindrosporum* might be more successful if a method were devised to purge the gut contents of infected larvae prior to isolating the fungus.

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Table 6.1. Percent mortality of *Aedes aegypti* exposed to different concentrations of conidia of *Tolypocladium cylindrosporum* serially passaged through mosquito larvae

	Concentration (conidia/ml)				
Passage	Control	10^3	10^4	10^5	10^6
0	6[1]	13	37	62	100
6	9	7	14	69	100
13	2	5	33	48	100
18	2	2	19	73	99

[1]Mean mortalities of 2 completely randomized assays. Each assay consisted of 3 replicates of 20 2nd instar larvae at each concentration/isolate combination.

7. Studies on bioassay of the entomopathogenic hyphomycete *Tolypocladium cylindrosporum* in mosquitoes[1]

7.1 INTRODUCTION

Bioassays allow for comparison of virulence of different species, strains, or batches, as well as susceptibilities of different hosts. Therefore, development of an adequate bioassay method is critical in the study of any potential microbial control agent.

Difficulties associated with bioassay of insect pathogens are well documented (Bucher and Morse 1963, Burges and Thompson 1971, Hall and Papierok 1982, Huber and Hughes 1984). To date only a few assay systems have been developed for fungi as there are many problems associated with their bioassay. The greatest assay problem involves our inability to deliver infectious propagules to the assay host in a standardized manner. The usual route of infection is *via* the exocuticle, but, in the mosquito pathogenic fungus, *Culicinomyces clavisporus* Couch, Romney and Rao, the primary route of infection is through the foregut and hindgut following ingestion of the conidia (Sweeney 1975). Sweeney (1976) was able to obtain adequate results with *C. clavisporus* based on the highly successful mosquito bioassay method for chemical insecticides (Brown and Pal 1971) and *Bacillus thuringiensis israelensis* (McLaughlin *et al.* 1984); namely exposure of mosquito larvae to different

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[1]A version of this chapter has been submitted for publication. M.S. Goettel. Entomophaga.

concentrations of test material in the rearing medium. Although Sweeney's (Loc. cit.) results were highly variable, the log dose, probit mortality regressions were generally linear.

Tolypocladium cylindrosporum Gams is a potential microbial control agent of mosquitoes. It causes epizootics in larval mosquito populations in New Zealand (Weiser and Pillai 1981) and California (Sanders' 1972, Soares *et al.* 1979). To date all mosquitoes challenged with *T. cylindrosporum* have been susceptible (Gardner 1984, Pinnock' *et al.* 1973, Soares 1979, 1982, Soares and Pinnock 1984, Weiser and Pillai 1981, Yu[2] *et al.* 1980). Preliminary evaluations of dosage responses indicated that probit mortality regressions were generally linear (Soares 1982).

This paper reports on dose-mortality assays conducted to quantify the pathogenicity of *T. cylindrosporum* conidia using larvae of *Aedes aegypti* (L.), *Ae. vexans* (Meigen), and *Culiseta inornata* (Williston).

7.2 MATERIALS AND METHODS

7.2.1 Mosquito colonies

A Florida strain of *Ae. aegypti* was obtained from the Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida and a colony of *Cs. inornata* was

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[1]Initially described as *Beauveria tenella* but later confirmed as *T. cylindrosporum* (Soares *et al.* 1979).
[2]Initially described as *Culicinomyces* sp. but later confirmed as *T. cylindrosporum* (J.S. Pillai, pers. comm.).

established from field-collected larvae and adults from the Edmonton area. All *Ae. vexans* larvae used were field-collected from the Devon, Alberta area.

7.2.2 Inoculum Preparation

Californian isolates of *T. cylindrosporum* [TC3=INRA 3 (Institute National Recherche Agronomique, La Miniere, France)= UAMH 4561 (University of Alberta Microfungus Collection and Herbarium)]; TC4=INRA 4=UAMH 5002) as well as isolate TC3 passaged through mosquito larvae 6 (TC3S6), 13 (TC3S13), and 18 (TC3S18) times (see chapter 6) were cultured for two weeks at 20°C on 50 ml Pablum mixed cereal agar (Padhye *et al*. 1973) or potato dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin (PDA-SP) in 200 ml culture flasks. Conidial suspensions were obtained by adding 100 ml of sterile distilled water to each culture flask and shaking for several minutes. Conidial counts were made using an improved Neubauer hemocytometer. Appropriate serial dilutions were made in Bates' medium S (McLintock 1952) to give final concentrations of 1 x 10^3, 10^4, 10^5, 10^6, and on several occasions, 10^7 conidia/ml.

Conidial viability was determined by spreading 0.1 ml of a 1 x 10^6 conidia/ml suspension onto the surface of PDA-SP in a standard Petri dish. Following incubation at 25°C for 24 hrs, the numbers of viable and non-viable conidia were counted on the plate surface under phase

contrast at a magnification of 200X. Conidia were considered viable if they swelled to a "barbell" or "peanut" shape. This swelling occurs just prior to the formation of a germ tube (see chapter 9). Three replicates were prepared for each assay. Counts were made of 5 fields of view per plate. Viabilities were always above 70% and in most cases, over 85%.

7.2.3 Assay Method

Batches of 20 2nd-instar larvae were added to 200 ml of each conidial concentration in 500 ml plastic containers (7 cm high x 11 cm wide; approx 3 cm water depth). A pinch of ground up Tetramin® fish food was sprinkled onto the surface of each container for larval food and was subsequently added as required. All containers were covered with sheets of glass to minimize evaporation and were kept in incubators at either 10, 15, 20, or 25°C. *Aedes aegypti* larvae were kept at a photoperiod of 12/12 while *Ae. vexans* and *Cs. inornata* were kept at 16/8 (L/D). Distilled water was added periodically to compensate for evaporation. Pupae were removed daily and were placed into multi-chambered trays (15 ml capacity/cell) until emergence. Adults were kept in the culture tray for at least 48 hrs after emergence. Dead insects were removed daily, stored at 4°C for 24 to 48 hrs and were then examined microscopically for mycosis (i.e. presence of hyphae in the hemocoel). Within 48 hrs of death, infected adults could be easily recognised by naked eye by

the appearance of a white cottony fungal growth on cadavers. Each assay consisted of 3 replicates at each of 4 or 5 concentrations and a control. A total of 37 bioassays consisting of different mosquito/temperature combinations were carried out.

Dose and time-mortality results of each assay were subjected to probit analysis (probit mortality and log dose or time) (Finney 1971) using the computer program of SAS Institute Inc., Cary, N.C. All mortalities were adjusted for control mortality using Abbott's (1925) formula. Time-infection mortality responses were also subjected to probit analysis (probit infection mortality as percent of total number infected and log time) to obtain estimated ST_{50}'s (survival time for 50% of total number infected). Statistically significant differences were judged by mutually exclusive 95% fiducial limits.

Infection rate data were analyzed by the one-way analysis of variance using angular transformation of proportions infected and log transformed numbers of individuals infected. These were further analyzed using Scheffé's test at the 95% level of significance (Sokal and Rohlf 1969).

7.3 RESULTS

All 3 test mosquito species were susceptible to the fungus; *Cs. inornata* is a new host record. Examples of time-dose-mortality responses obtained for each species are

given in Figs. 7.1-7.3. Control mortalities in *Ae. vexans* and *Cs. inornata* were high. *Aedes aegypti* proved to be a better bioassay organism because of lower control mortalities, but control mortality was 100% in this species at 10°C. There was generally a direct relationship between dosage of conidia and mortality in the host population; however, there was much variability. In some instances higher mortalities were obtained than at the next higher dose (i.e. see Fig. 7.2). Eleven of the 37 bioassays performed showed linear relationships between probit mortality at 10 days and log dose (Table 7.1). Mortalities taken at 20 days were even less amenable to probit analysis as in most of these, mortalities of 100% were obtained at the highest concentrations, while at the lowest concentration the mortalities were lower or equal to the control mortality. In such cases, there were only two points on which to base the dose-mortality regression line. In the assays where probit analysis was possible on 15 and 20 day mortality, the LC_{50}'s did not differ significantly from those at day 10.

There were significant differences in the LT_{50}'s between replicate assays (Table 7.2). For instance, for *Ae. aegypti* with isolate TC3 at 20°C the variance comprised large, significant between-assay components (5.99, 7.72) and small, within assay components (0.11,0.06) (for 10^5 and 10^4 conidia/ml respectively), thereby, contributing to the very wide 95% fiducial limits of the weighted means. The LT_{50}'s

increased with a decrease in temperature for *Ae. vexans* and *Cs. inornata*, although, this effect was not as apparent in *Ae. aegypti*. Within each assay the LT_{50}'s were higher at 10^3 than at 10^4 conidia/ml except for *Ae. vexans* at 10°C. In many of the assays, control mortalities were much too high to yield any meaningful LT_{50} estimates. This mostly occurred at the lowest temperatures with all species and at the highest temperature with *Ae. vexans* (Table 7.3).

As in the LT_{50} estimates, there were significant differences in the ST_{50} estimates between replicate assays (Appendix 2). The ST_{50}'s followed much the same pattern as the LT_{50}'s with 15 of 21 assays having no significant differences between the two estimated parameters at 10^4 conidia/ml. The ST_{50}'s increased with a decrease in temperature in all cases at 10^3 conidia/ml. At 10^4 conidia/ml, the two exceptions were with isolate TC3 and *Ae. vexans* and *Ae. aegypti* with no differences between 25 and 20°C. The ST_{50}'s were also higher at 10^3 than at 10^4 conidia/ml except for *Ae. aegypti* with isolates TC3 at 25°C and TC3S13 at 20°C where no differences were apparent.

Tolypocladium cylindrosporum was infectious to mosquitoes at all temperatures tested (Table 7.3). Mosquitoes succumbed to the fungus at all developmental stages tested, with 90% of the total number infected in all assays (n=1584) dying as larvae, 9% as pupae, and 1% as adults. For the 6 assays with *Ae. aegypti* and isolate TC3 at 20°C, there were significant differences in the mortality

rates of the uninfected individuals at the different doses (Fig. 7.4). The control, 10^3 and 10^4 conidia/ml treatment uninfected mortalities were the same, whereas, significantly more individuals died without any signs of mycosis at 10^5 and 10^6 conidia/ml (Fig. 7.4). This indicates that at the 2 lowest doses all mortality over and above control mortality can be attributed to mycosis. However, there were no significant differences in the proportions of infected to uninfected individuals between each dose.

7.4 DISCUSSION

Difficulties were encountered in bioassay of *T. cylindrosporum* due to variability in the responses, the majority of which were not amenable to probit analysis. Similar results were obtained by Soares (1979, 1982). In his studies with *T. cylindrosporum* against *Ae. sierrensis* Ludlow, 4 of 28 assays conducted were amenable to probit analysis; however, initial assays were hampered by poor yields and clumping of spores. Of 4 successful assays, 2 were heterogenous. At certain times in both of his assays with conidia, higher mortalities occurred than at the next higher dose [i.e. higher mortality at 5 x 10^6 than at 5 x 10^7 conidia/ml for assay 3 and at 5 x 10^5 than at 5 x 10^6 conidia/ml in assay 4 (Soares 1979, Figs. 32 and 33, see also Soares *et al.* 1979)]. Clearly, the bioassay method whereby mosquito larvae are continuously exposed to different concentrations of *T. cylindrosporum* is inadequate

and needs major improvement.

7.4.1 Sources of variation

Differences between batches of insects and pathogen, as well as inaccuracies in the estimation of the dose are usually the principal sources of variation in bioassays (Bucher and Morse 1963, Burges and Thompson 1971). However, these are usually sources of variation that occur between assays. In the present bioassay of *T. cylindrosporum*, the major variability of response is occurring within assays as well as between assays. Since the assay method reported in this paper is similar to the one used successfully for *C. clavisporus*, the difficulties associated in the assay of *T. cylindrosporum* will be discussed relative to the assay of *C. clavisporus*.

With *C. clavisporus* the primary route of infection is through the gut, but at very high concentrations, infection can occur through the anal papillae; however, this is a relatively rare occurrence (Sweeney 1979). With *T. cylindrosporum* the infection sites are through the external cuticle, pharynx, and midgut (Soares 1982); however, the relative importance of each site is not known. It is conceivable that the primary sites of infection may be affected by factors such as dose, species, and length of exposure.

Since the assay system used is a closed one, larvae are continuously exposed to the inoculum. Gardner (1984)

reported 90% conidial viabilities of *T. cylindrosporum* which had been held for 9 months in distilled water at 25°C. I have shown that ingested conidia of *T. cylindrosporum* are still viable when excreted (chapter 9). Therefore, the effective dose will vary according to the length of exposure as mosquitoes are reingesting the conidia again and again. Furthermore, since the ingestion of conidia is presumably not contributing to the nutrition of the mosquito, it is probable that as the concentration of the conidia is increased, the larvae at each higher concentration are put under increasing stress as they ingest more and more non-nutritive matter (i.e. conidia) and thereby become more susceptible to mycosis. In his bioassays, Soares (1979, 1982) used autoclaved conidia equivalent to the highest concentration in the assay as well as water with no conidia as controls, however, he did not report any differences in the mortalities between these two. It is not known if mosquitoes are able to digest autoclaved conidia. Boiled conidia of *C. clavisporus* did not inflict significant mortalities in *Ae. aegypti* and *Anopheles hilli* Woodhill and Lee (Panter and Russell 1984, Sweeney 1983).

The bioassay is further complicated since each assay cup rapidly becomes a habitat of its own with a different microbial fauna. After a few days it was common to observe different turbidities among the assay containers. Such differences came about due to random contamination by microorganisms from the air and due to larval mortalities

that subsequently affected the amount of food in each container. The abundance, nature, and size of particulate matter in turn affect larval ingestion rates (Dadd 1970a, b), thereby presumably affecting the amount of inoculum ingested. In addition the microbial fauna most likely has a effect on the viability of the conidia. Consequently, in this type of closed bioassay system, longer exposure times will tend to increase variability between replicates and doses.

LT_{50}'s of *T. cylindrosporum* conidia are approximately 4 to 8 times greater than those of *C. clavisporus* (Sweeney, 1983) while LC_{50}'s are greater by factors of 10 to 100 (Cooper and Sweeney, 1982). Sweeney (1976) found that his data were highly heterogenous when 2nd instars were used; heterogeneity was reduced when very young 1st instar larvae were used. The reduction of heterogeneity was probably a result of the more uniform physiological state of the younger larvae as well as their decreased median lethal time (Sweeney, 1983). Subsequently, the assay method was further improved by using mortalities at 4 days rather than at 7 days (Cooper and Sweeney, 1982). Dose-mortality responses of different instars to conidia of *T. cylindrosporum* have not been studied, although, Pinnock *et al.* (1973) demonstrated that younger instars were more susceptible to blastoconidia.

7.4.2 Possible improvements in assay technique

Since in *T. cylindrosporum* there is generally a direct relationship between dosage of conidia and mortality in the host population, it should be possible to develop an adequate bioassay by decreasing sources of variability. Possible ways to accomplish this would be to; 1) use very young larvae in order to decrease variability among them 2) inoculate each assay container with a standard suspension of bacteria in an attempt to standardize the microbial fauna 3) expose larvae to the inoculum for a limited time in order to standardize the dose as was done in the bioassay of *Nosema* (Jaronski 1979) and 4) increase the number of doses so that the mortalities are evenly distributed around the LC_{50}'s.

Even though the assay method was unsuccessful as far as studying the relative potency of *T. cylindrosporum*, much useful information was gained. In the assays that were amenable to probit analysis, the estimated median lethal concentrations and times were similar to those reported for *Ae. sierrensis* (Soares 1982, Soares and Pinnock 1984). Microbial control agents of mosquitoes with long LT_{50}'s are deemed beneficial as it allows for intraspecific competition pressure to act on the uninfected individuals (Barr 1985). However, in *T. cylindrosporum* it is not known if the relatively long LT_{50} is a result of a slow infection rate or due to infections being acquired at a later date.

7.4.3 Host death and fungal colonizaton of hemocoel

At the lower doses, mortality rate of uninfected individuals was the same as for the control. At higher doses many more larvae died without fungal colonization of the hemocoel. It appears the increase in dosage of conidia has a detrimental affect on mosquito viability without fungal invasion of the hemocoel. Since dead larvae were removed daily, it is possible that those larvae exhibiting mycosis were the ones that had died shortly after the last collection while those not exhibiting mycosis had died shortly before removal. This would imply that death usually occurs before invasion of the hemocoel; however, Soares (1982) noted that infected larvae generally did not die until hyphae packed a major portion of the body. In his infection studies with *T. cylindrosporum*, all moribund 3rd and 4th instars of *Culex tarsalis* Coquillett and *Ae. sierrensis* that he examined were infected, often quite extensively. It is unlikely that in the present study such mycoses were overlooked. On the other hand, it is possible that the mode of pathogenesis varies among different species of mosquito.

The presence of ungerminated conidia in the guts of mosquitoes has been reported to be detrimental (Batchinsky 1927, Panter and Russell 1984, Roberts 1970). In *C. clavisporus* death occurs within hours of ingestion of large quantities of conidia (Panter and Russel 1984). It was speculated that death was the result of release of toxins by

germinating conidia. In the present study, however, deaths with no invasion of the hemocoel occurred throughout the exposure period. Furthermore, since there were no differences in the proportions of dead immatures with and without mycosis between the different doses (Fig. 7.4), this phenomenon was occurring at the same rate at all doses.

7.4.4 Summary

It can generally be concluded that *T. cylindrosporum* is a relatively slow acting mosquito pathogen of low virulence which requires massive doses to elicit a response. Attempts to further evaluate *T. cylindrosporum* as a microbial control agent of mosquitoes are presently hampered by the lack of an adequate bioassay method. A better understanding of the host-pathogen relationships is needed.

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Table 7.1. Probit analysis of dose-mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum*

Mosquito Species	Fungus Isolate	Temp. °C	Assay[1] #	LC_{50} (x10⁶conidia/ml)	95% Fiducial Limits	Chi²(df)	H²	Slope±S.E.
Ae. vexans	TC3	20	18	2.8	1.0-5.5	1.05(2)	+	1.14±0.218
Ae. aegypti	TC3	25	15	67.6	42.3-108.5	5.00(3)	+	1.01±0.114
			21	15.2	6.6-46.8	3.11(2)	+	0.49±0.086
		20	18	6.5	3.9-10.4	2.02(2)	+	1.17±0.159
			21	3.4	0.2-39.5	4.95(2)	-	0.98±0.177
		15	15	44.5	23.8-81.2	5.75(3)	+	0.78±0.105
	TC4	20	16	5.6	3.6-8.5	3.96(2)	+	1.41±0.181
	TC3S6	20	21	5.4	3.8-7.9	2.00(2)	+	1.44±0.154
	TC3S13	20	16	1.2	0.7-1.9	1.63(2)	+	1.16±0.147
	TC3S18	20	20	0.7	0.5-1.0	0.18(1)	+	1.36±0.165
			21	6.7	4.6-9.7	1.10(1)	+	1.54±0.182

[1]Each assay consisted of four dose levels with 60 larvae/dose. Mortalities were taken after 10 days of continuous exposure of second instar larvae and were corrected for control mortality using Abbott's formula (Abbott 1925).
[2]Within assay homogeneity.

Footnotes for Table 7.2

[1]Mortalities were corrected for control mortalities on each day using Abbott's formula (Abbott 1925).

[2]Each assay consisted of continuous exposure of 60 2nd instar larvae per dose.

[3]Fiducial Limits.

[4]Control mortality at 10 days.

[5]Not done.

[6]Calculated according to Bliss (1952).

Table 7.2. Probit analysis of time-mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum*

Mosquito Species	Fungus Isolate	Temp. °C	Assay[1] #	LT_{50} (ix10[3]	95% Limits[2] conidia/ml)	LT_{50} (ix10[4]	95% Limits[2] conidia/ml)	% Control Mortality[4]
Ae. vexans	TC3	20	18	7.2	7.0-7.5	6.2	6.0-6.4	43
		10	14	13.5	13.1-13.8	13.4	13.0-13.8	33
Cs. inornata	TC3	20	07	13.6	12.6-15.0	nd[3]	-	17
			10	12.7	12.2-13.2	8.6	8.0-9.1	27
			11	10.0	9.5-10.6	nd[3]	-	23
		Weighted Mean[4]:		12.1	7.5-16.8			
		15	10	mort.<50%		13.8	13.3-14.3	20
Ae. aegypti	TC3	25	15	mort.<50%		7.4	7.0-7.8	2
			21	mort.<50%		6.5	6.0-7.0	3
		20	12	mort.<50%		7.6	7.2-7.9	8
			15	10.1	9.6-10.5	6.8	6.1-7.4	10
			16	14.0	13.1-15.3	11.9	11.2-12.7	2
			18	8.4	8.0-8.9	7.4	7.1-7.7	8
			20	mort.<50%		3.3	2.6-3.7	8
			21	9.6	9.2-10.0	6.0	5.6-6.3	2
		Weighted Mean[4]:		10.5	6.5-14.4	7.1	4.2-10.1	
		15	12	mort.<50%		8.7	8.0-9.2	20
			15	11.9	11.6-12.2	7.9	6.7-8.8	7
	TC4	25	19	mort.<50%		6.2	5.2-7.2	7
		20	16	9.3	8.9-9.7	3.9	3.4-4.2	5
			21	mort.<50%		6.2	5.9-6.5	2
	TC3S6	20	20	12.0	11.3-12.8	4.2	3.9-4.6	2
			21	10.1	9.7-10.5	6.0	5.7-6.3	0
	TC3S13	20	16	8.0	7.7-8.3	7.3	7.1-7.5	12
			20	mort.<50%		5.8	5.5-6.0	2
			21	10.7	10.2-11.3	5.0	4.6-5.3	2
		Weighted Mean[4]:		-	-	6.0	3.1-8.9	
	TC3S18	20	20	10.1	10.0-10.6	6.5	6.2-6.8	0
			21	9.4	9.1-9.8	6.4	6.1-6.7	0

Table 7.3. Mortality rate and proportion of dead larvae with diagnosed mycosis after exposure to different concentrations of *Tolypocladium cylindrosporum* conida for 20 days at different temperatures

Mosquito Species	Fungus Isolate	Temp. °C	No. of Assays[1]	% Mortality (Proportion infected)[2] at Each Dose[4]					
				0	10^3	10^4	10^5	10^6	10^7
Cu. inonnata	TC3	20	3	48(0)	69(6)	73(28)	90(60)	97(79)[5]	nd[6]
		15	3	28(0)	19(0)	28(19)	51(64)	67(72)[5]	nd[6]
		10	1	15(0)	15(0)	8(0)	12(0)	72(41)	nd[6]
Ae. vexans	TC3	25	1	92(0)	93(0)	95(0)	97(5)	100(33)	100(18)
		20	2	60(0)	61(16)	65(18)	70(42)	97(59)	98(59)[5]
		15	1	70(0)	73(0)	68(0)	86(36)	100(50)	98(39)
		10	1	50(0)	50(0)	53(0)	92(5)	98(29)	100(15)
Ae. aegypti	TC3	25	2	2(0)	20(25)	22(55)	41(67)	67(58)	83(30)[5]
		20	6	9(0)	16(34)	26(57)	63(56)	93(54)	100(78)[5]
		15	2	26(0)	24(7)	47(25)	72(62)	99(47)	97(38)[5]
		10	2	100(0)[5]	100(0)[5]	90(11)[5]	97(31)	99(31)	100(82)[5]
	TC4	25	2	22(0)	27(22)	22(46)	34(51)	72(38)	nd[6]
		20	4	20(0.3)	32(47)	62(48)	70(43)	97(30)	nd[6]
	TC3S6	20	2	8(0)	6(0)	13(75)	69(83)	100(62)	nd[6]
	TC3S13	20	3	9(0)	15(36)	44(57)	62(59)	99(52)	nd[6]
	TC3S18	20	2	2(0)	2(0)	19(55)	73(74)	99(76)	nd[6]

[1]Pooled data.
[2]Each assay consisted of continuous exposure of 60 2nd instar larvae per dose.
[3]Proportion of deads infected= # with diagnosed mycosis/total # dead x 100.
[4]Conidia/ml.
[5]Results of one assay.
[6]Not done.

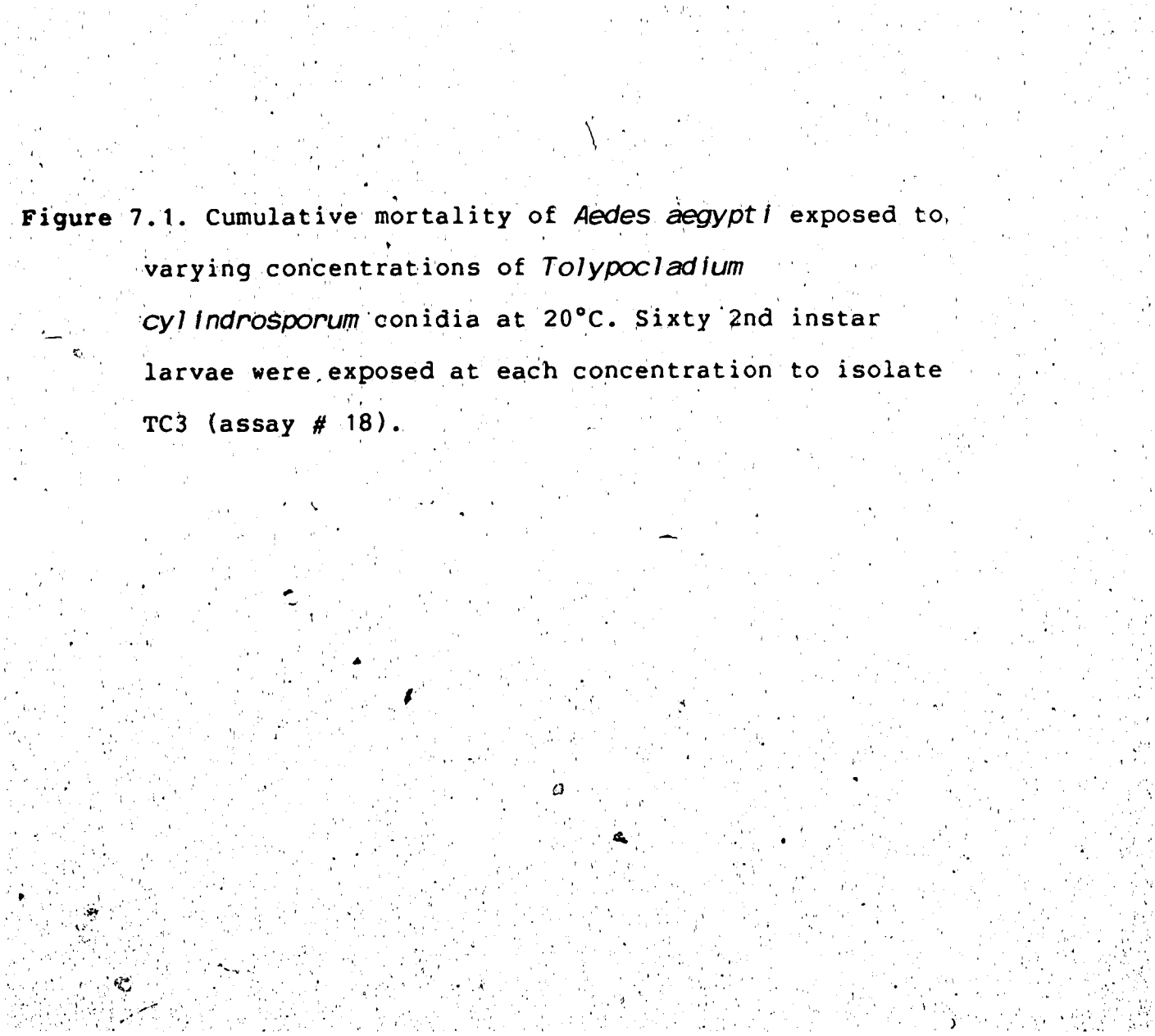
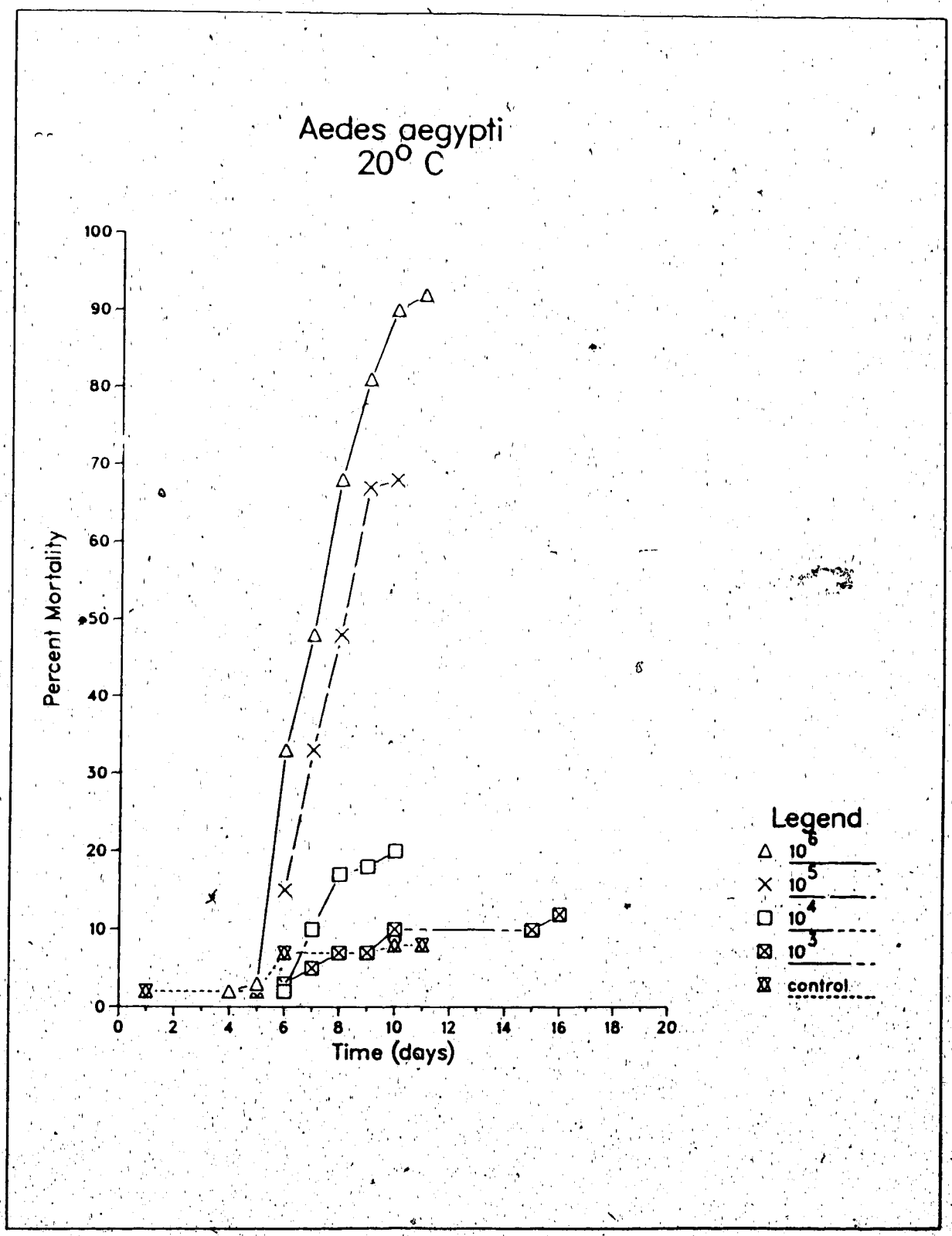


Figure 7.1. Cumulative mortality of *Aedes aegypti* exposed to varying concentrations of *Tolypocladium cylindrosporum* conidia at 20°C. Sixty 2nd instar larvae were exposed at each concentration to isolate TC3 (assay # 18).


Aedes aegypti
20° C
Percent Mortality
Time (days)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
Legend
10^6
10^5
10^4
10^3
control

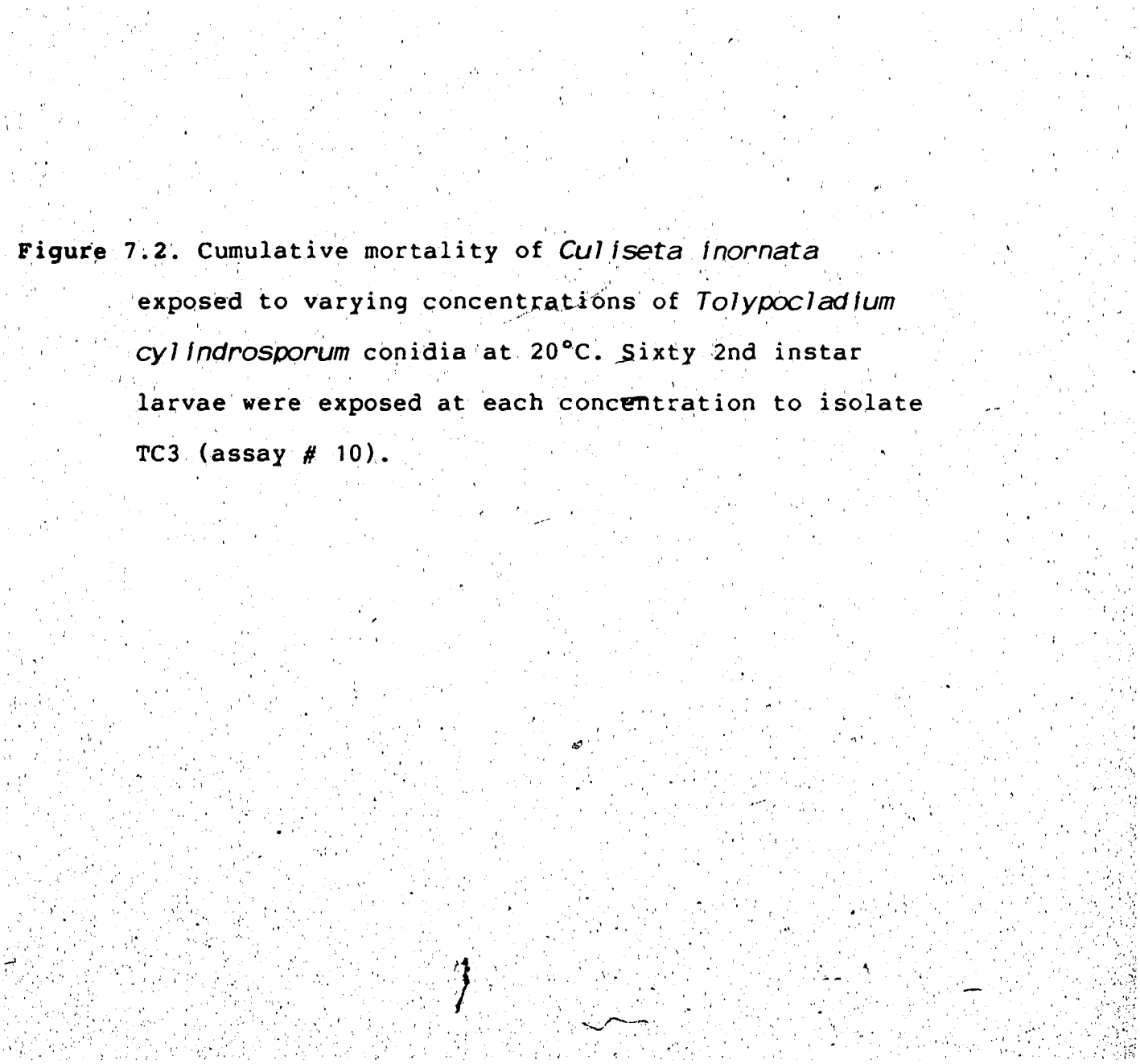


Figure 7.2. Cumulative mortality of *Culiseta inornata* exposed to varying concentrations of *Tolypocladium cylindrosporum* conidia at 20°C. Sixty 2nd instar larvae were exposed at each concentration to isolate TC3 (assay # 10).

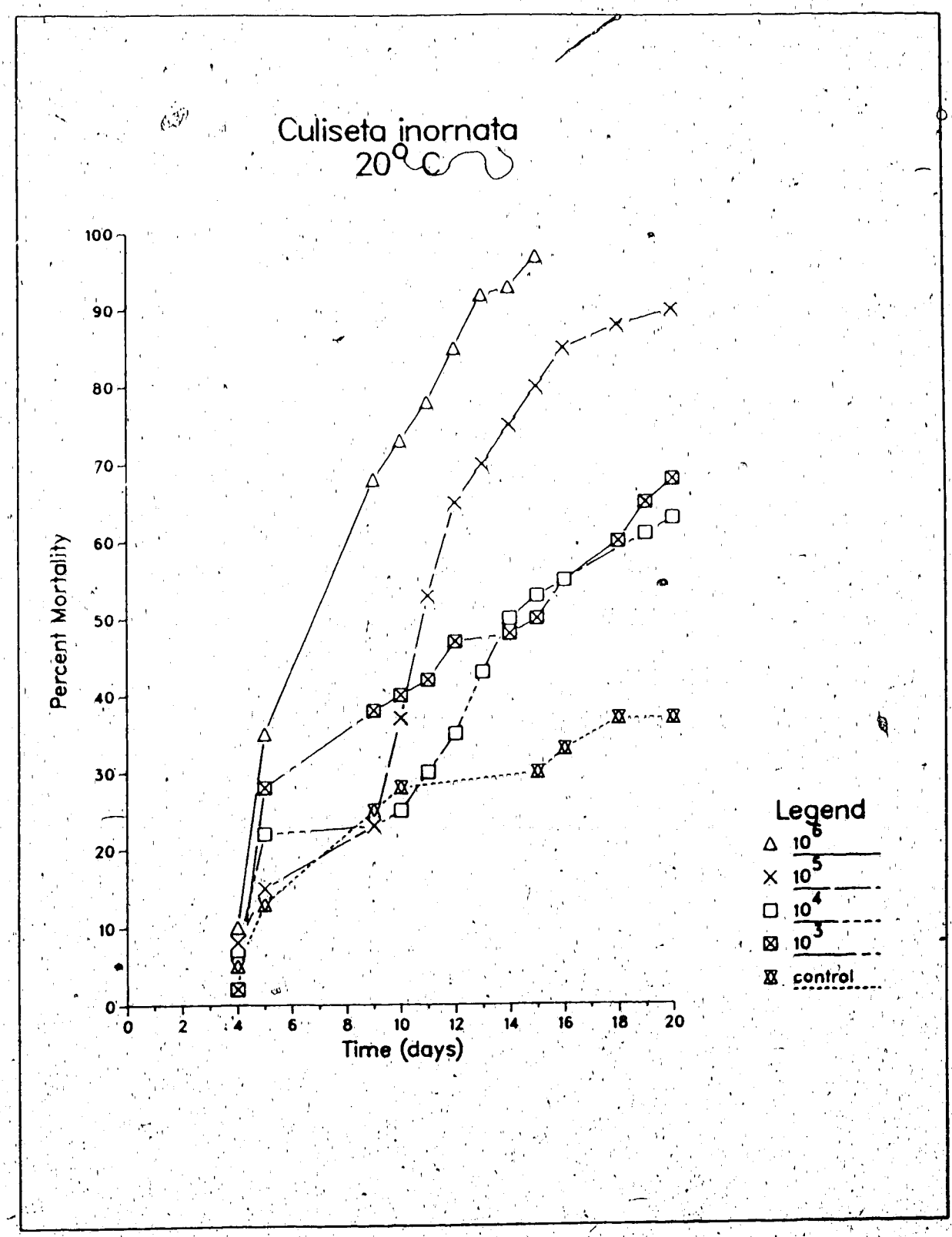

Culiseta inornata
20°C
Percent Mortality
Time (days)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
Legend
10^6
10^5
10^4
10^3
control

Figure 7.3. Cumulative mortality of *Aedes vexans* exposed to varying concentrations of *Tolypocladium cylindrosporum* conidia at 20°C. Sixty 2nd instar larvae were exposed at each concentration to isolate TC3 (assay # 18).

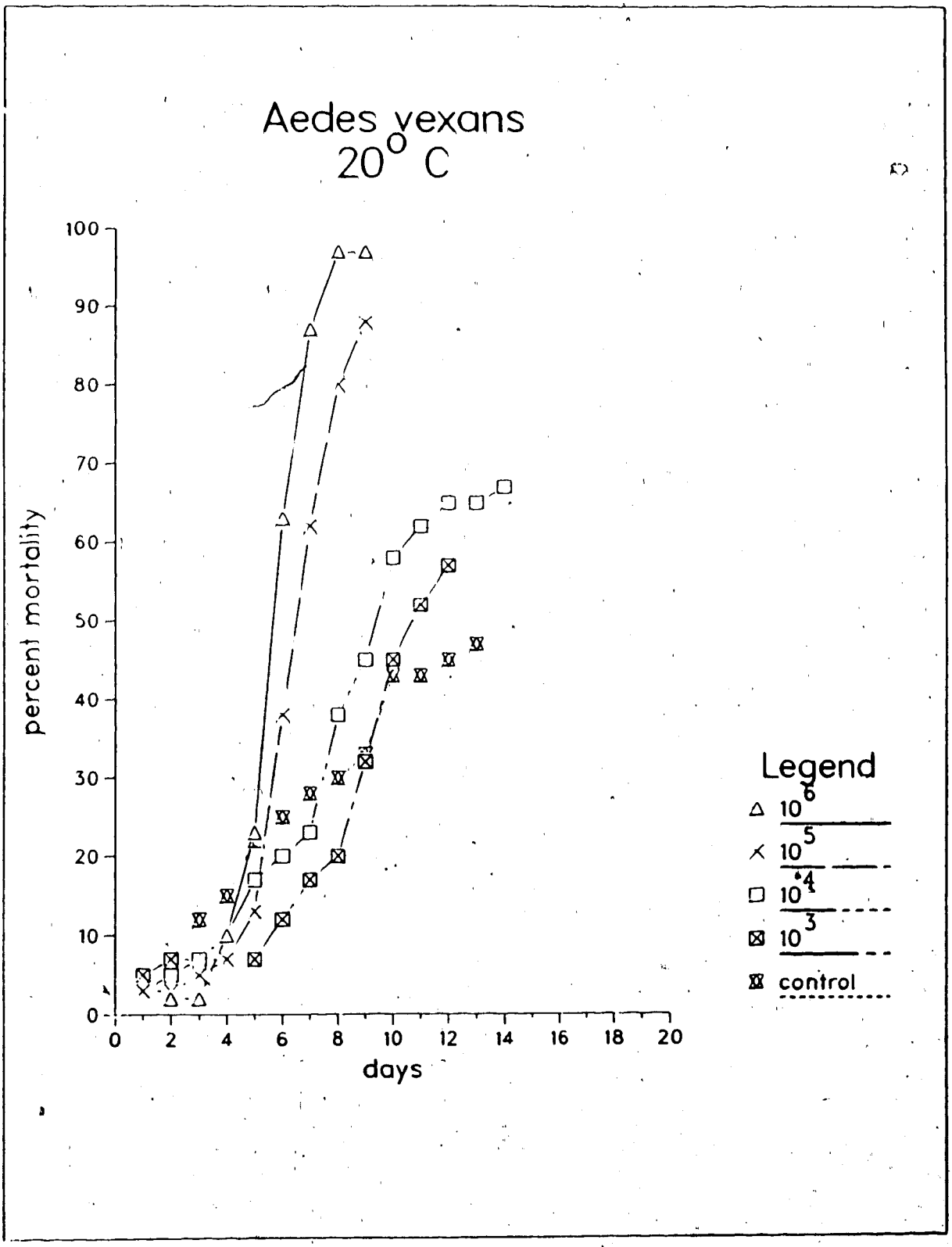
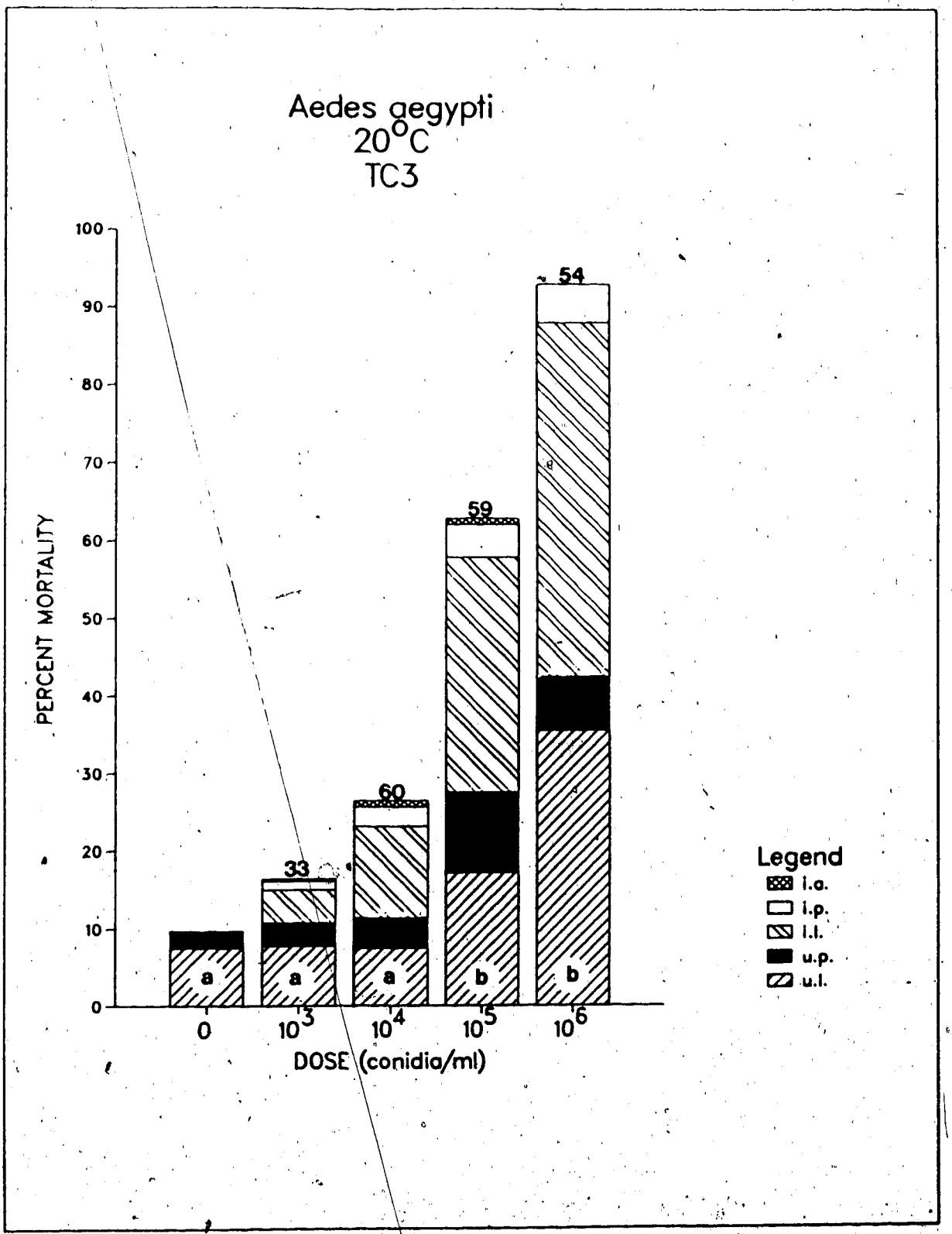

Aedes vexans
20° C
percent mortality
days
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
Legend
10^6
10^5
10^4
10^3
control

Figure 7.4. Mortality of *Aedes aegypti* exposed to conidia of *Tolypocladium cylindrosporum* at 20°C in relation to stage, infection diagnosis, and dose. Data pooled from 6 assays of 60 larvae/dose/assay with isolate TC3. Numbers above columns refer to mean proportion of total dead with diagnosed mycosis (%). There were no significant differences between these means ($F= 0.86$; $p= 0.48$). Letters within columns refer to significant differences between the mean mortalities of uninfected immatures ($F= 5.5$; $p= <0.003$) as determined by Scheffé's test at the 95% level of significance. i.a. = infected adults, i.p. = infected pupae, i.l. = infected larvae, u.p. = uninfected pupae, u.l. = uninfected larvae.


Aedes aegypti
20°C
TC3
PERCENT MORTALITY
100
90
80
70
60
50
40
30
20
10
0
54
59
60
33
a
a
a
b
b
0
10³
10⁴
10⁵
10⁶
DOSE (conidia/ml)
Legend
i.a.
i.p.
i.l.
u.p.
u.l.

8. Preliminary field trials with the entomopathogenic hyphomycete *Tolypocladium cylindrosporum* against mosquitoes in central Alberta[1]

8.1 INTRODUCTION

Tolypocladium cylindrosporum Gams is a candidate as a microbial control agent of mosquitoes. It causes epizootics in larval mosquito populations in New Zealand (Weiser and Pillai 1981) and California (Sanders[2] 1972, Soares *et al.* 1979). To date all species of mosquitoes challenged with *T. cylindrosporum* have been susceptible (Gardner 1984, Pinnock[2] *et al.* 1973, Soares 1979, Soares *et al.* 1985, Weiser and Pillai 1981, Yu[3] *et al.* 1980, also see chapter 7); they include 10 species in the genus *Aedes*, 6 in *Culex*, 2 in *Culiseta*, and 1 each in *Anopheles*, *Maorigoeldia*, and *Opifex*.

Introduction of blastoconidia (also referred to as "blastospores" or "hyphal bodies") of *T. cylindrosporum* into tree holes in California resulted in reductions of up to 71% in the emergence of *Aedes sierrensis* (Ludlow) (Pinnock *et al.* 1973). Introduction of conidia into crab holes in Fiji resulted in reductions of 87% of immature *Ae. polynesiensis* Marks (Gardner *et al.* 1986), but applications of blastoconidia to ground pools in New Zealand were not as

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[1]A version of this chapter has been submitted for publication. M.S. Goettel. J. American Mosquito Control Association.
[2]Initially described as *Beauveria tenella* but later confirmed as *T. cylindrosporum* (Soares *et al.* 1979).
[3]Initially described as *Culicinomyces sp.* but later confirmed as *T. cylindrosporum* (J.S. Pillai, pers. comm.).

successful (Gardner and Pillai 1986).

This paper reports on field applications of *T. cylindrosporum* conidia and blastoconidia to semipermanent and temporary ground pools in central Alberta conducted to further evaluate this fungus as a microbial control agent of mosquitoes.

8.2 MATERIALS AND METHODS

8.2.1 Inoculum Preparation, Storage, and Application

A Californian isolate of *T. cylindrosporum*, INRA 3 (Institute National Recherche Agronomique, La Miniere, France)= UAMH 4561 (University of Alberta Microfungus Collection and Herbarium) was used. Blastoconidia were produced by inoculating 500 ml of Sabourauds dextrose broth in 1 l Nalgene® flasks with 1 ml of a 1 x 10^6 conidia/ml suspension. The cultures were incubated at 20°C and agitated at approximately 170 oscillations/min on a Burrell wrist action shaker for 4 - 5 days. Yields were typically in the order of 10^7 blastoconidia/ml.

For trials 1 through 5, conidia were produced on the surface of Pablum mixed cereal agar (Padhye *et al.* 1973) in large Petri dishes (14 cm diam) or in tin trays (36 x 26 x 4 cm). These were inoculated with a conidial suspension and were incubated at 20°C for 2 weeks. Conidia were harvested by washing off with sterile distilled water. These were then applied in the field within 4 hours of harvest.

For trial 6, conidia were produced on cellophane surface and a wheat bran medium according to the method outlined in chapter 4. The cellophane, with adhering fungal mat, was rolled up, placed in plastic bags, and frozen at -20°C for later use. Conidia were stockpiled over an 11 month period using approximately 24 pans in continuous culture.

For field application, the conidia which had been frozen on the cellophane surface were thawed, scraped, and then washed off. The resultant pulp was homogenized in a blender for approximately 5 min. The 262 pans yielded 18 l of a 9 x 10⁹ conidia/ml suspension which was the equivalent of approximately 1 kg dry weight of conidia. Prior to field application, samples of the inoculum were tested for viabilty and bioassayed (see chapter 7)[1]. Viability was always above 80%.

The fungus was applied as a coarse spray to grassy roadside ditches on six separate occasions (Table 8.1) using a 20 l capacity back-pack sprayer equipped with a hand pump. Because of difficulties in culturing adequate amounts of inoculum, preliminary trials consisted of applications to portions of pools. Trials 1, 2, and 4 consisted of applications to different portions of semipermanent pools (25 x 50 m for trials 1 and 4; 25 x 2 m for trial 2). Trials 3 and 5 consisted of applications to different portions of a

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[1]The assay numbers in chapter 7 corresponding to the field trials are as follows: assay 07 for field trial 4 and assay 18 for trial 6. The assay for trial 4 was omitted due to very high mortalities at all concentrations on day 1.

temporary pool (30 x 50 m). Trial 6 consisted of application to the entire pool.

8.2.2 Larval monitoring and diagnosis

Approximately 12 pools and ponds 4 km NW of Devon, Alberta (114°47'W, 53°23'N) were continuously monitored over a three year period as part of a study to evaluate the incidence of naturally occurring mosquito pathogens in central Alberta. Some of these study pools were also used to evaluate field applications of *T. cylindrosporum*.

The pools were monitored from mid-April until the end of October. Ten to 30 samples were taken from each pool on a weekly basis using a 350 ml capacity dipper. Sampling was increased to every 2 to 4 days following application of the fungus. During each field trip, pool parameters such as water temperature, pH, and conductivity were monitored.

Field collected immatures (i.e. larvae and pupae) were brought back to the laboratory, counted, and placed into Bates' medium S (McLintock 1952) in trays or 500 ml plastic containers. They were reared at 20°C until emergence or death. Adults were held for at least 48 hrs post emergence and were then identified using the key of Wood *et al.* (1979). Dead larvae were removed daily, identified using the same key, and then examined microscopically as whole wet mounts for mycosis (i.e. presence of mycelia in the hemocoel). At times, dead immatures were stored at 4°C for 24 - 48 hrs prior to examination.

In 1982, 10 sentinel cages were placed into the water immediately after fungus application. Ten to 25 larvae that had been collected prior to treatment were then placed into each cage. Five cages were also placed in a control pool. The sentinel cages were constructed from 1-l plastic tubs (10 cm diam x 13 cm depth). These had 4 3-cm diam holes cut at equal distances in the side and 1 6.5-cm diam hole each in the lid and bottom. All holes were screened with a nylon mesh (13 holes/cm). The cages were floated with Styrofoam® so that 8.5 cm of the cage was left submerged.

Sentinel cages were examined during each visit to the pools. The number of mosquitoes present was recorded and any dead immatures were brought back to the laboratory where they were examined microscopically as whole wet mounts for mycosis. Pupae and missing larvae were replaced with freshly collected larvae from a control pool.

During the summer of 1982, all individuals with *T. cylindrosporum* mycosis were bathed in a 50 µg/ml sterile solution of chloromycetin for 5 - 10 minutes and then placed in Petri dishes containing potato dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin. These were incubated for 2 weeks at 20°C after which they were examined for the characteristic white cottony growth of *T. cylindrosporum*. To verify the identity of the fungus, slide cultures were prepared for each field isolation. As diagnostic experience was gained, this procedure was discontinued after the first year.

8.2.3 Infection rate estimates

Because large numbers of mosquitoes were unaccounted for while reared under laboratory conditions, two methods of estimating infection rates of field-collected mosquitoes were used (see also chapter 2).

(1) Minimum estimated rate of infection= number infected/total collected x 100.

In this calculation, it is assumed that none of the missing immatures were infected and therefore the absolute minimum infection rate possible is estimated.

(2) Maximum estimated rate of infection= estimated number infected/total collected x 100.

The estimated number infected was determined by multiplying the proportion of accounted dead immatures with infection times the total number collected less the number of adults emerged. In this calculation, it is assumed that the same proportion of missing immatures were infected as those that were accounted for. This calculation also takes into account the number of adults emerging.

Where possible, the results of the field data were subjected to statistical analysis. Time-infection mortality responses were subjected to probit analysis (probit infection mortality as percent of total number infected and log time) (Finney 1971) using the computer program of SAS Institute Inc., Cary N.C., to obtain estimated ST_{50}'s (survival time for 50% of total number infected). Statistically significant differences were judged by

mutually exclusive 95% fiducial limits. Infection rate data were analyzed by the one-way analysis of variance using angular transformation of proportions infected and log transformed numbers of individuals infected. These were further analyzed using Scheffé's test at the 95% level of significance (Sokal & Rohlf 1969). Only the results of dip samples where the number of immatures were 25 or greater, were used for statistical analysis (in trial 6, n=12 for day 3, 11 for day 5, and 10 for day 8). In trial 6 only 4 samples were taken twelve days post application, therefore, the results of this collection were excluded from statistical analyses.

8.3 RESULTS AND DISCUSSION

8.3.1 Mosquito prevalence and composition

In 1982, the pools in which trials 1, 2 and 4 were carried out contained larvae from the first day of monitoring (15 June) until 6 Oct. In 1983, larvae were present from 5 May until 22 Sept. In 1984, larvae were present only between 12 June and 10 July; thereafter, the pools dried up.

The pool in which trials 3, 5, and 6 were carried out was colonized by snow melt *Aedes* in the early spring (April-May) of all three years. Subsequently, large numbers of *Ae. vexans* (Meigen) hatched after heavy rains in early to mid-summer. In 1982 this occurred in the first week of July.

Subsequently, the pool was recolonized by *Culiseta inornata* (Williston) and *Culex territans* Walker in early August until drying late that month. In 1983 the pool was flooded in the third week of June and the pool became colonized by *Ae. vexans* until mid-July. Thereafter the pool remained without mosquitoes until drying in the first week of August.

In 1984 the pool was flooded between 9 and 6 days prior to treatment. A large hatch of *Ae. vexans* resulted. A second rainfall two days prior to treatment produced a second smaller hatch. At the time of treatment the mean water depth was 10 cm and it rapidly decreased so that by day 15, the pool was virtually dry.

8.3.2 Control estimates using mosquito densities

Tolypocladium cylindrosporum conidia are very slow acting with LT_{50}'s of 3 - 14 days (Soares and Pinnock 1984, also see chapter 7). Although long LT_{50}'s are deemed a beneficial attribute of potential microbial control agents of mosquitoes (Barr 1985), such pathogens pose unique problems in their field evaluation. In the present study, the efficacy of *T. cylindrosporum* could not be determined based on comparisons of mosquito densities in treatment and control pools due to fluctuating mosquito densities, water volumes, and uneven maturation rates. Detailed results of larval densities are therefore not presented.

Similar difficulties were experienced by Gardner and Pillai (1986) in field evaluations of *T. cylindrosporum* in

New Zealand and by Sweeney (1982) with *Culicinomyces clavisporus* Couch, Romney and Rao in Australia. Sweeney (1982) experienced such large variability in mosquito densities even between mosquito populations in identical artificial ponds started simultaneously that he concluded "there would be little value in designating control sites for future field tests."

8.3.3 Control estimates using sentinal cages

Difficulties arose with the use of sentinel cages. These often became rapidly invaded by dense mats of algae and, at times, predators such as flatworms were also found in them. Also, the cages were sometimes invaded by first instar larvae as the mesh size was not small enough to exclude them.

Mortalities in control and treatment sentinel cages were similar. In trial 3, however, 2 days post application, there was a significant reduction in the number of larvae in the treatment cages as compared to the control (30% reduction corrected by Abbott's (1925) formula for control mortality) ($F=9.81$, $p=0.0079$). Subsequently, there were no differences in the mortality rates between cages in control and treatment pools.

The use of sentinel cages demonstrated that the application of *T. cylindrosporum* blastoconidia resulted in infections and mortality of mosquitoes in the field. Dead infected larvae were found in sentinel cages only in trial 3

as follows: 2 days post application, 2 dead larvae (1% of mosquitoes accounted for in all traps); 4 days, 1 dead larva (0.5%); 6 days, 10 dead larvae (5%) (with 9 larvae found infected in a single cage) and 10 days, 1 dead larva (1%). Subsequently, no infected larvae were found for the 26 days that the cages remained in operation.

8.3.4 Control estimates using field-collected larvae

Because of high mortalities under laboratory conditions in both treatment and control pool-collected mosquitoes, control estimates could not be made using these parameters. Also, many immatures went missing while held in the laboratory. In collections where infections of *T. cylindrosporum* were noted, up to 66% of immatures collected were unaccounted for from the time of collection to the time the last individual either died or emerged as an adult (mean±S.E.=33±4.7, n=18). Similar rates of unaccounted immatures were occurring in the collections from the control pools (max 56%, mean±S.E.=33±6.1, n=10). Presumably this was largely a result of cannibalism and rapid decomposition of dead immatures held in the laboratory.

8.3.5 Incidence of mycosis in field-collected immatures

Much information was gained by examining all dead individuals microscopically for mycosis. Applications of either blastoconidia or conidia under field conditions resulted in infections of 5 species of mosquitoes

subsequently reared under laboratory conditions; *Cx. territans* and *Cu. minnesotae* Barr are new host records. Of the 937 mosquitoes diagnosed with mycosis, 0.1% were 2nd instars, 19% were 3rd, 80% were 4th, 10% were pupae and 0.2% were adults. Similar rates of infection at each stage were occurring when 2nd instar larvae were exposed to conidia in the laboratory (see chapter 7).

In trials 1 to 5, infections occurred in collections up to 29 days post application (Table 8.2). No infections were detected in the monitored pools during subsequent years. Estimated maximum infection rates for any collection date ranged from 0 to 33% for blastoconidia and 0 to 28% for conidia. Since applications of the fungus in these trials were made to portions of pools, it is felt that these estimates are conservative because of probable immigration and emmigration of larvae from the spray zone area.

In trial 6, maximum infection rates for any collection date ranged from 17 to 50%. There were no significant differences in the maximum and minimum estimated rates of infection between the collections of 3, 5, and, 8 days post application ($F=0.38$, $p=0.69$; $F=1.18$, $p=0.32$, respectively). However, there were significant differences in the numbers of accounted mosquitoes that died in the laboratory ($F=3.9$, $p=0.03$) (mean±S.E. on day 3=93±9.0; day 5=82±12.5 and day 8=65±15.5%) with significantly less mortality on day 8 than day 3. On day 12, 86% (n=468) of the accounted mosquitoes died in the laboratory.

In laboratory bioassays, I demonstrated that large numbers of immatures died after exposure to conidia of *T. cylindrosporum*, but without fungal colonization of the hemocoel; between 6 and 79% of dead immatures were found with infection at various doses (chapter 7). In the present study, between 20 and 54% of the dead mosquitoes died in the laboratory with mycosis; there were no significant differences in these proportions between the collections of day 3, 5, and, 8 (day 3=29±5.3%; day 5=20±4.6; and day 8=24±7.7) ($F=0.62$; $p=0.54$). On day 12, the rate was 55% (n=419 in one dip) for trial 6 and 28.2±5.12 (n=84) for trial 5. Based on the laboratory findings, it can be assumed that in the present study, immatures also died as a result of exposure to *T. cylindrosporum* but without fungal colonization of the hemocoel. Therefore, the estimated infection rates in the field-collected mosquitoes are probably only a portion of the total mortality that could be attributed to the fungus.

8.3.6 Epizootiology

Natural epizootics of *T. cylindrosporum* have been reported to occur in mosquitoes only at relatively low temperatures. Weiser and Pillai (1981) observed the fungus in *Ae. australis* (Erichson) in supralittoral pools on the coast of Otago, New Zealand "in the same pool every year in July and August." At that time of year, pool temperatures are between 5 and 10°C (Gardner 1984). In California,

epizootics were reported in larvae of *Ae. sierrensis* breeding in tree holes between March and April when the water temperatures are 11 - 13°C (Soares 1979). Although epizootics resulting in up to 91% mortality have been reported (Sanders 1972), the epizootiology remains unclear as infections were diagnosed only after holding larvae in the laboratory. Weiser and Pillai (1981) reported "samples of population brought to the laboratory will develop the infection and larvae are killed." Soares (1982) reported "samples brought back to the laboratory in early March and incubated at 18 - 20°C often showed mortality due to this fungus in excess of 70%." In field studies in New Zealand, Gardner and Pillai (1986) were unable to detect larval mortality in the field after application of *T. cylindrosporum* blastoconidia against larvae of *Ae. subalbirostris* Klein and Marks at water temperatures of 4 to 10°C or against larvae of *Ae. australis* at water temperatures of 16 to 20°C. However, samples of larvae brought back to the laboratory and kept at 15°C or placed on Sabourauds agar plates at 25°C developed growth of *T. cylindrosporum*.

Although there have been no reports of *T. cylindrosporum* epizootics (i.e. large numbers of dead infected mosquitoes) occurring directly in the field, one cannot conclude that epizootics do not occur. For instance very few dead larvae were collected following application of *C. clavisporus* in Australia even though reductions of larval

numbers in the order of 80 - 100% were recorded (Sweeney *et al*. 1983). It was speculated that many of the dead specimens sank to the bottom and were not recovered by the dipper. In the present study infections were detected in the field only following application of blastoconidia; in trial 3, two larvae were found infected 4 days post application. Infections were also detected in sentinel cages.

Indications are that infections did not occur in the field following application of conidia; infections were detected only after holding the larvae for 2 - 24 days under laboratory conditions (Table 8.2). Further evidence of this can be found in the median survival times of the infected larvae (Table 8.3). Although there were statistically significant differences in the ST_{50}'s between the different collection dates (Table 8.3), indicating that the infection had progressed to a certain degree in the field, (i.e. in trial 6, day 12), these differences are probably insignificant since these ST_{50}'s are within the same range as those of 2nd instar larvae exposed to conidia under laboratory conditions (Appendix 2).

In the present study, water temperatures were low (10 - 22°C). Median lethal times for conidia at these temperatures are in the order of 10 to 22 days (Soares and Pinnock 1984, also see chapter 7). These low temperatures may be responsible for the lack of infection mortality in the field. Once larvae were brought back to the laboratory and placed at 20°C, the infection process was initiated.

For blastoconidia, LT_{50}'s are between 1 and 5 days for *Ae. sierrensis* at temperatures encountered (Soares and Pinnock 1984); however, in the present study, larvae collected 28 and 29 days post application of blastoconidia succumbed to the fungus after being held in the laboratory for up to 12 days. It is highly unlikely that blastoconidia survived for up to 29 days under field conditions for it is generally believed that they are short-lived (Ferron 1981, Roberts and Humber 1981). Soares and Pinnock (1984) observed that blastoconidia germinated and produced conidia after prolonged incubation at 25°C. It is possible that this also occurred in the present field trials. This would explain the length of persistence in the field as well as the time that it took for larvae to succumb in the laboratory, since conidia are slower acting. It is also possible that the larvae became infected much earlier in the field, but these infections remained latent until the larvae were brought under laboratory conditions.

Laboratory studies have shown that relatively high dosages of *T. cylindrosporum* are required to elicit a response in mosquitoes (Pinnock *et al.* 1973, Soares 1982, also see chapter 7). It is highly unlikely that such high doses would occur in the field under natural conditions. Sporulation on mosquitoes occurs only on floating cadavers and only conidia are produced (Soares 1982). Blastoconidia are only produced in the hemolymph of infected larvae or in liquid broth culture.

Present evidence suggests that very few larvae succumbed to the fungus under field conditions even after application of massive doses. Since *T. cylindrosporum* is a common soil inhabitant (Bissett 1983), conidia may be washed into pools after heavy rains and then ingested by mosquitoes. Larvae containing these conidia in the gut and subjected to stress such as low temperature or transfer to laboratory conditions may then succumb to the fungus. In the present study, field-collected larvae placed under laboratory conditions suffered considerable stress as evidenced by the high mortality rates in the controls. Presumably, larvae in other studies also suffered the same type of stress.

Even if mosquitoes succumb to *T. cylindrosporum* only as a result of stress, this should not preclude its potential as a microbial control agent. With proper manipulation of stress factors, it might be possible to use microorganisms that are not normally highly pathogenic for pest control(Steinhaus 1960). Thorough understanding of the fungus-host relationships is required in order to further evaluate *T. cylindrosporum* as a microbial control agent of mosquitoes.

8.4 BIBLIOGRAPHY

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Table 8.1. Field applications of *Tolypocladium cylindrosporum* against mosquitoes in central Alberta

Trial #	Date	Inoculum	Species composition (%)	Density[1]	Surface area (m²)	Rate[2] (per m²)	Temp °C	pH	Cond[3]
1	24/vi/82	blastoconidia	*Cs. alaskaensis* (90) *Cs. morsitans* (3) *Cs. inornata* (5) *Cx. territans* (2)	7	5	2.5×10^9	16	7.3	640
2	1/vii/82	blastoconidia	*Cs. alaskaensis* (8) *Cs. morsitans* (8) *Cx. territans* (83)	2	5	3.9×10^9	15	7.4	460
3	17/vii/82	blastoconidia	*Ae. vexans* (99) *Cx. territans* (1)	213	20	1.5×10^9	13	7.6	375
4	23/viii/82	conidia	*Cs. inornata* (90) *Cs. morsitans* (6) *Cx. territans* (4)	9	6	1×10^{11}	14	8.7	850
5	23/vi/83	conidia	*Ae. vexans* (100)	246	0.7	5×10^{11}	16	8.7	74
6	14/vi/84	conidia	*Ae. vexans* (100)	212	150	1×10^{11}	22	7.1	50

[1]Mean no. of immatures per 350ml dip.
[2]Rate of application for portion of pool treated.
[3]Conductivity in micromhos/cm at 20°C.

Table 8.2. Effects of field applications of *Tolypocladium cylindrosporum* against mosquitoes in central Alberta

Trial #	Collection time[1]	Density[2] (instar)	Species infected (no.)	Infection rate[3]	Time of death[4]
1	29	0.1 (all)	*Cu. minnesotae* (1)	17-33	12
2	5	3 (all)	*Cx. territans* (1)	3	9
	7	2 (all)	*Cx. territans* (3)	14-18	11-18
	28	3 (all)	*Cx. territans* (1)	2-3	12
3	2	126 (3)	*Ae. vexans* (8)	2	2-16
	4	125 (3-4)	*Ae. vexans* (7)	1-2	0-7
	6	58 (4)	*Ae. vexans* (3)	1	5-6
	10	38 (4)	*Ae. vexans* (2)	1	3
4	2	16 (all)	*Cu. inornata* (1)	1	16
	4	8 (all)	*Cx. territans* (1) *Cu. inornata* (1)	2-3	4-9
	8	8 (all)	*Cu. inornata* (4)	3-4	8-13
5	5	45 (3)	*Ae. vexans* (1)	1	9
	12	46 (4)	*Ae. vexans* (28)	12-20	5-19
	19	21 (4-p)	*Ae. vexans* (1)	1	3
6	3	113 (2-4)	*Ae. vexans* (392)	14-35	3-16
	5	76 (2-4)	*Ae. vexans* (182)	10-23	4-24
	8	54 (2-p)	*Ae. vexans* (65)	6-17	2-19
	12	109 (2-p)	*Ae. vexans* (229)	30-50	2-9

[1]Days post treatment
[2]Mean # of immatures per 350ml dip.
[3]Infection rate of field collected immatures reared under laboratory conditions; range of estimated infection = # infected/total collected x 100 to # estimated infected/total collected x 100: see text for details.
[4]Death attributed to mycosis in days post collection.

Table 8.3. Probit analysis of time-infection mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum* under field conditions and subsequently reared in the laboratory at 20°C

Field trial	Collection time[1]	ST_{50}[2]	95% Limits[3]	Range[4]
5	12	9.2	8.7-9.8	5-19
6	3	5.3	5.1-5.5	3-16
	5	6.6	6.2-7.0	4-24
	8	6.3	5.8-6.8	2-19
	12	4.3	4.2-4.5	2-9

Probit analysis of cumulative daily infection mortality as percent of total number infected and log time.
Infection mortality= # individuals with diagnosed mycosis.
[1]Days post application.
[2]ST_{50}=Survival time for 50% of total number infected.
[3]Fiducial Limits.
[4]Range between first and last infection mortality in days.

9. Pathogenesis of the hyphomycete *Tolypocladium cylindrosporum* in the mosquito *Aedes aegypti*

9.1 INTRODUCTION

Tolypocladium cylindrosporum Gams is currently being investigated as a potential microbial control agent of mosquitoes. The effects of pH, temperature, and salinity on *T. cylindrosporum* germination and growth are well documented (Soares and Pinnock 1984, Gardner and Pillai 1986a), but conidial germination and initial ramification of hyphae have not been previously described. *Tolypocladium cylindrosporum* causes epizootics in larval mosquito populations in New Zealand (Weiser and Pillai 1981) and California (Sanders' 1972, Soares *et al.* 1979), although mass applications of conidia failed to induce epizootics in field populations of mosquitoes in New Zealand (Gardner and Pillai 1986b) and in Alberta (chapter 8).

Larvae can become infected via the exocuticle, pharynx, or midgut (Soares 1982); however, the importance of each site in pathogenesis was not previously studied. Also, many larvae die without fungal colonization of the hemocoel when exposed to high concentrations of conidia (chapter 7). Previously, the pathogenesis of *T. cylindrosporum* in these larvae was not known. Furthermore, bioassays whereby larvae were continuously exposed to different concentrations of *T. cylindrosporum* conidia proved highly variable (Soares 1982,

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'Initially described as *Beauveria tenella* but later confirmed at *T. cylindrosporum* (Soares *et al.* 1979).

Chapter 7). Clearly, a better understanding of the pathogenic processes of *T. cylindrosporum* in mosquitoes is needed before this fungus can be considered further as a potential microbial control agent of mosquitoes.

In the present study a series of experiments were carried out to determine the pathogenesis of *T. cylindrosporum* in larvae of *Aedes aegypti* (L.). Host death in relation to larval feeding, molting, and fungal colonization of the hemocoel, the principle sites of infection, and viability of gut-passaged conidia, were determined. In addition, observations were made on conidial germination, fungal growth and sporulation on surface or in submerged culture using scanning electron and light microscopy.

9.2 MATERIALS AND METHODS

9.2.1 Mosquito colony

A Florida strain of *Aedes aegypti* was obtained from the Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida.

9.2.2 Inoculum preparation

A Californian isolate of *T. cylindrosporum* [TC4=INRA 4 (Institute National Recherche Agronomique, La Miniere, France)= UAMH 5002 (University of Alberta Microfungus Collection and Herbarium)] was cultured for two weeks at

20°C on potato dextrose agar supplemented with 60 µg/ml penicillin and 30 µg/ml streptomycin (PDA-SP) in standard Petri plates. Conidia were washed off the plate surface with 10 ml of sterile distilled water and conidial concentrations were determined using an improved Neubauer hemocytometer. Appropriate dilutions were made in Bates' medium S (BMS) (McLintock 1952) to give a final concentration of 1 x 10^4 conidia/ml. Blastoconidia were produced by inoculating 500 ml of Sabouraud dextrose broth in 1 l Nalgene® flasks with 1 ml of a 1 x 10^4 conidial/ml suspension. The cultures were incubated at 20°C and agitated at approximately 170 oscillations/min on a Burrell wrist action shaker for 4 - 5 days. Yields were typically in the order of 10^7 blastoconidia/ml. For blastoconidial germination studies, the cultures were filtered through Whatman® no. 1 filter paper under gentle vacuum to remove hyphae. The filtrate was centrifuged at 2000 rpm for 30 minutes, washed twice in distilled water, recentrifuged, and then resuspended in BMS,

9.2.3 Fungal growth and development

Conidial germination, fungal growth and sporulation on surface or in submerged culture were examined by scanning electron and light microscopy. A 0.1 ml conidial suspension was placed onto a 6 cm cellophane square on the surface of Sabouraud dextrose agar in a standard Petri plate and spread with a glass rod. Three replicate plates were prepared and these were incubated at 20° C. A one cm square of cellophane

was cut out at 4 hr intervals and examined under phase contrast at a magnification of 200x and then vapour-fixed for 24 - 48 hr by placing in a chamber containing 5 ml of 3% osmium tetroxide. The squares were air-dried for 72 hrs and mounted on SEM stubs using conductive silver paint. Using SEM, conidiogenesis was also observed on infected cadavers kept in a humid chamber for 4 days. Parallel observations on conidiogenesis were made in slide culture on cereal agar (Padhye *et al*. 1973). For submerged culture studies, inocula were prepared in distilled water, BMS, and in Sabouraud dextrose broth. Samples were removed from these media every 4 hr and examined under phase contrast.

9.2.4 Viability of gut-passaged conidia

To enable monitoring of ingestion of conidia, mosquitoes were first glutted with ink by placing in a sterile solution of india ink (3 drops/100 ml water) for 24 hr at 25° C. Batches of 10 larvae were then transferred to 100 ml of a 1 x 10^4 solution of conidia in 200 ml flasks. After 1.5 hr all the ink within the larval guts was displaced by conidia. At this time, the larvae were transferred to 10 ml of fresh india ink solutions in 20 ml test tubes. After 1 hr the conidia within the larval guts were displaced by the ink and the larvae were removed. The ink solution containing the excreted conidia was centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 1 ml of the supernatant by mixing on a vortex for 2 min. The

number of conidia per larva and conidial viability were then determined. Groups of conidia-glutted larvae were also held in BMS for 2.5 hr prior to purging as described above.

Conidial counts were made using an improved Neubauer hemocytometer. Conidial viability was determined by spreading 0.1 ml of a 1 x 10^4 conidia/ml suspension onto the surface of PDA-SP in a standard Petri plate. Following incubation at 25°C for 24 hrs, the numbers of viable and non-viable conidia were counted on the plate surface under phase contrast at a magnification of 200X. Conidia were considered viable if they swelled to a "barbell" or "peanut" shape.

Viability of conidia prior to exposure to larvae; held in water for 2.5 hr; ingested and excreted by larvae; and ingested, held in the gut for 2.5 hr, and then excreted by larvae were determined and analyzed by the one-way analysis of variance (arcsine transformed % viabilities). There were 5 replicates of each treatment with 10 larvae per replicate and the whole experiment was replicated twice, once with 2nd instar larvae and once with 3rd instars.

9.2.5 Mode of infection

Batches of 20 2nd-instar larvae were added to 200 ml of inoculum in 500 ml plastic containers (7 cm high x 11 cm wide; approx 3 cm water depth). Containers were covered with sheets of glass and kept in an incubator at 20°C at a photoperiod of 12/12. A pinch of ground Tetramin® fish food

was added as required. Distilled water was added periodically to compensate for evaporation. Several larvae were removed every 12 hr and examined under the microscope as whole wet mounts prior to fixation. Three containers were kept as controls and another 3 served to monitor host mortality and incidence of mycosis.

Larvae were fixed either in Baker's formalin (Humason 1967) or in 3% gluteraldehyde for 24 hr followed by 24 hr in 1% osmium tetroxide, dehydrated in a graded ethanol series to xylene, and embedded in Tissuemat®. Serial sections 7 μm thick were stained by the periodic acid method of DePalma and Young (1963). Whole larvae were also examined by SEM.

Techniques for examining the interior of larvae by SEM were adapted from Armstrong (1971). Serial sections of the gluteraldehyde/osmium fixed specimens were cut to the desired depth. The remaining portion of the specimen in the wax block was cut out and deparaffinized by 4 changes of warm toluene (55°C) followed by 2 changes of warm absolute alcohol.

In addition, dead larvae obtained during bioassay studies (chapter 7) were observed under phase contrast as whole wet mounts. Records were kept of the areas in which hyphae could be seen within the hemocoel. Larvae not colonized by the fungus were fixed in formalin, sectioned, and stained as described above.

Terms used in the description of the proventriculus are those used by Romoser and Venard (1966).

9.2.6 Scanning electron microscopy

Specimens were mounted on SEM stubs using double-sided sticky tape, air-dried for 72 hr, sputter coated with gold in a Nanoteck-Semprep 2, and observed on a Cambridge S 250 SEM operated between 10 - 20 kV.

9.2.7 Host-parasite interactions

Second instar larvae were placed singly into 10 ml of inoculum in multi-chambered trays (15 ml capacity/chamber). Twenty-four larvae were placed with blastoconidia and 30 with conidia. Larvae were fed 2 drops of a sterile baker's yeast-Tetramin® solution (1 gm each in 1 l water) every second day and were examined every 3 hr between 9 AM and 9 PM. At least once each day each larva was placed in the ink solution for 1 hr, observed under the microscope at 200x and then returned to its original cell. Times of molting; feeding and voiding of ink; presence of hyphae in the hemocoel; and time of death were recorded. Pupae were transferred to fresh BMS and adults were observed for up to 72 hr after emergence.

I refer to the molting period as the time after apolysis to immediately following ecdysis but prior to full sclerotization of the cuticle. Larvae were considered to be immediately prior to ecdysis if lateral hairs or pupal respiratory siphons could be observed in the thorax.

Secondly, 2nd-instar larvae, purged of conidia as described above, were placed into 10 ml of a 1 gm/l solution

of kaolin for 3 - 4 hr to purge the ink which may have contained reingested conidia, and then were transferred to 100 ml BMS. A 0.1 aliquot of the kaolin/ink mixture was plated onto PDA-SP and observed for fungal growth after 48 hr incubation at 25°C. Larvae were also added to the conidial suspension at day 0 and into a suspension that had been held for 7 days at 25°C. Larvae were also kept in BMS as controls.

Larvae were fed initially with 10 ml, and every second day thereafter with 1 ml of the yeast-Tetramin solution. Mortality was recorded daily and whole wet mounts of dead larvae were examined microscopically for presence of hyphae in the hemocoel. There were 5 replicates of each treatment with 10 larvae per replicate. Final mortalities were analyzed by the one-way analysis of variance (arcsine transformed % mortalities). These were further analyzed using Scheffé's test at the 95% level of significance (Sokal and Rohlf 1969).

9.3 RESULTS

9.3.1 Fungal growth and development

Tolypocladium cylindrosporum produces cylindrical phialidic conidia aerially which accumulate in slimy masses (Fig. 9.1). Blastoconidia (also referred to as "blastospores" or "hyphal bodies") are borne terminally or on short pegs from hyphae (Fig. 9.2) and are produced within

the host hemocoel or in liquid shaker culture. On one occasion I observed what appeared to be phialidic conidium development within the hemocoel of an infected mosquito cadaver (Fig. 9.3).

No visible changes occurred in blastoconidia prior to germination (Fig. 9.4). Blastoconidia germinated within 4 hr; hyphae grew and ramified (Fig. 9.5) and attained lengths of approximately 0.3 mm within 10 hr and sporulated in liquid culture within 22 hr (Fig. 9.2).

In contrast, conidia swelled to a "barbell" or "peanut" shape prior to germination (Fig. 9.6). This usually occurred within 24 hr of incubation on all media except distilled water. Conidia did not swell in distilled water for the 72 hr they were observed. Shortly after conidial swelling, a germ tube emerged from one or both ends of the conidium (Figs. 9.6, 9.7) and the resultant hypha grew and ramified and attained a length of approximately 0.2 mm on solid media and 0.4 mm in liquid culture in 48 hr. At this time the site of the original conidium could still be identified (Fig. 9.8). Blastoconidia were produced in liquid culture within 48 hr and conidia on solid media in 2 - 4 days. In slide culture, the fungus often formed club-shaped structures adpressed to the glass (Fig. 9.9). Similar structures were sometimes produced adpressed to the body wall within cadavers (Fig. 9.10).

Conidial germination and subsequent blastoconidial production were sparse in BMS but continued for at least 7

days. Viabilities of 96% were recorded up to 16 days at 25°C, but it was not possible to distinguish between the newly formed blastoconidia and the original phialidic conidia. Conidial germination in BMS also occurred in containers to which larvae were added, but observations on fungus development were not possible within 48 hr of the addition of larvae due to the rapid growth of a microbial fauna which resulted after larvae were added.

9.3.2 Viability of gut-passaged conidia

Ingestion and excretion of conidia did not affect conidial viability ($F=1.46$, $p=0.26$ for 3rd instars and $F=1.07$, $p=0.37$ for 2nd instars) (Table 9.1). Conidium-glutted, 2nd instar larvae contained approximately $5.6\pm0.95 \times 10^4$ conidia in their gut while 3rd instars contained $1.1\pm0.27 \times 10^5$ conidia. Conidium-glutted, 3rd instar larvae, held in BMS for 2.5 hr, excreted an estimated 25±8.0% of the conidia during this time.

9.3.3 Host mortality

Final mortalities of larvae exposed to *T. cylindrosporum* conidia were 90 - 100%; although, in one experiment, a final mortality of 64±10.1% was obtained (Table 9.2). There was much variability in mortalities of larvae exposed to blastoconidia (42 - 100%). Mortalities of larvae reared singly were 58% for blastoconidia and 96% for conidia. These were similar to mortalities of larvae reared

in groups of 20 (42% for blastoconidia; 98% for conidia).

There were no statistically significant differences between mortalities of control larvae and those which were purged of conidia (Table 9.2); however, 3 larvae in the purged group succumbed to the fungus 2 to 3 days after purging and contained extensive mycelial growth within the hemocoel. The kaolin/ink mixture which was excreted by the purged larvae and plated onto PDA-SP developed an abundant growth of *T. cylindrosporum* indicating that purging of conidia was not complete.

There were no statistically significant differences between mortality rates of the larvae added to the conidial solution at the commencement of the experiment and those added to conidia which were held for 7 days.

9.3.4 Host-parasite interactions

SEM observations of larvae periodically removed from the inoculum revealed that conidial attachment on the host surface was sparse. Conidia were most prevalent in folds and were present up to 8 days post inoculation (Figs. 9.11, 9.12).

Within 1 hr of exposure to conidia, fecal pellets consisted predominantly of conidia, excreted either loosely or enveloped in the peritrophic membrane (Figs. 9.13 - 9.16). Fecal pellets excreted 4 days after larval exposure to conidia and plated onto PDA-SP produced abundant growth of *T. cylindrosporum* in 48 hr.

Observations of larvae reared singly revealed that complete passage of the food column usually occurred within 1 hr. Feeding was interrupted and the gut was voided at least 2 hr and up to 14 hr prior to a molt. Feeding usually ceased 5 to 72 hr (most commonly 24 hr) prior to death. Fifty-two percent of the cadavers (n=42) were from larvae which had died during the molting period.

9.3.4.1 Host death prior to fungal colonization of hemocoel

Between 10 to 50% of immatures died without fungal colonization of the hemocoel. Between 70 and 80% of these (n=45) died during the molting period. In 64% of the cadavers examined without fungal colonization of the hemocoel (n=45), penetration of host tissues occurred (Table 9.3). In 36% of the cadavers there was no fungal penetration of the host; in 19%, conidia, germinating conidia, and hyphal pieces were present in the midgut and colon and in 17%, extensive mycelial growth occurred in the foregut, midgut, colon or rectum but without penetration of host tissues. Germination usually occurred while the gut contents were being voided prior to a molt (Figs. 9.17 - 9.20).

9.3.4.2 Host death after fungal colonization of hemocoel

Between 50 to 90% of the cadavers were colonized by *T. cylindrosporum* prior to death. Of these, 42% died during the molting period (Table 9.3). Host death usually occurred

within 24 hr but up to 48 hr after infection was first noticed. During this time period, major portions, and in many cases the whole hemocoel, was heavily packed with hyphae (Figs. 9.21, 9.22). Some larvae were diagnosed with infection 22 hr prior to cessation of feeding while others were diagnosed with infection up to 19 hr post cessation of feeding.

Tolypocladium cylindrosporum had ramified throughout the hemocoel in 48% of the larvae with mycosis that were obtained during bioassay studies (see chapter 7). In the remainder, hyphae were localized within the hemocoel; 48% were localized in the head/thorax area, 25% in the posterior abdomen (generally in the area of the saddle/anal papillae, Fig. 9.22) and 27% in both areas. Head/thorax localizations were more prevalent in 2nd instars and decreased in each subsequent instar (Fig 9.26). Head/thorax infections were also most prevalent in mosquitoes exposed to 10^4 conidia/ml (Fig. 9.27).

9.3.5 Infection sites

9.3.5.1 Head

The head was the principle infection site. Infections invariably originated at the base of the mandibles or more often the maxillae (Figs. 9.23 - 9.25). Germinating conidia or blastoconidia within the bolus often penetrated the pharynx in individuals that were molting, but this was

usually a secondary infection site (Fig. 9.25). In one instance, infection occurred at the apex of the antenna (Fig. 9.28).

9.3.5.2 Thorax

Infections in the thorax usually occurred only after extensive invasion had already taken place in either the head or posterior abdomen. Infections of the thorax originated in the esophagus within the proventriculus. The fungus penetrated the cuticle of the esophagus and grew anteriorly between the epidermal and annular muscle cells of both the esophagus and reflected esophagus (Figs. 9.29 - 9.31). Hyphae growing between the annular muscle and the epidermis of the reflected esophagus emerged in a ring around the muscular connective (Fig. 9.32). In one instance, hyphae growing between the annular muscle and the epidermis of the esophagus appeared to be in the process of penetrating back into the esophagus (Figs. 9.33, 9.34).

9.3.5.3 Abdomen

Localized mycoses in the abdomen were observed most often in the area of the saddle and anal papillae (Fig. 9.22). At times this was the primary infection site, although, most often it was accompanied by a more extensive mycosis in the head. Heavy melanization of invading hyphae was sometimes observed especially in the posterior abdomen (Figs. 9.35, 9.36). Penetration usually occurred at the junction of the anus and anal papillae; however, the precise

area of penetration was difficult to determine in most specimens (Fig. 9.37). Penetration was also observed from the rectum (Fig. 9.38) and less often from the colon.

Germination of conidia and blastoconidia within the midgut and hindgut usually occurred only in larvae that were in the molting period or that were already extensively infected either in the head or posterior abdomen or in both these areas. The peritrophic membrane offered some protection as mycelial growth was usually initially confined within the food column, but fungal penetration into the ectoperitrophic space was often observed (Figs. 9.39 - 9.41). Penetration of the midgut cells, ileum, or colon was rare and usually occurred only after an extensive mycosis had established elsewhere or if the larva was moribund (Fig. 9.42).

9.3.5.4 External cuticle

Penetration of the external cuticle occurred mostly in molting larvae (Table 9.3). *Tolypocladium cylindrosporum* sometimes penetrated the cuticle into the ecdysial space and began penetrating the underlying new cuticle (Fig. 9.43). In one instance, a hypha was seen penetrating into the hemocoel in the thorax of a moribund infected larva (Fig. 9.44). Penetration of the exuvium was often observed (Fig. 9.45).

9.4 DISCUSSION

The results of the present study demonstrate some complexities of the host-parasite relationships between *Ae. aegypti* and *T. cylindrosporum*: 1. *T. cylindrosporum* has multiple infection sites which differ according to the growth stage of the host, 2. larvae seem most susceptible during their molting period, and 3. conidia germinate and produce blastoconidia in the mosquito rearing medium.

9.4.1 Infection sites

Soares (1982) found that *T. cylindrosporum* penetrates larvae of *Ae. sierrensis* through the external cuticle as well as from within the alimentary tract. In the present study similar observations were made with *Ae. aegypti*, although it was found that the importance of each infection site differs. The two primary infection sites leading to a mycosis were at the base of the mandibles or maxillae and in the anal region, most probably at the junction of the anal papillae at the anus. Conidia were present on the external cuticle immediately after larvae were added to the inoculum; however, germination and penetration generally occurred in healthy larvae only in the above two regions.

The wax layer of the epicuticle has been reported to have antifungal properties (Koidsumi 1957, Wada 1957, Saito and Aoki 1983, Smith and Grula 1982). It is possible that the thin membranous cuticle at the base of the mouthparts and at the junction of the anal papillae at the anus is

devoid of antifungal substances which may be present elsewhere on the cuticle. Conidia could become lodged in these areas of articulation and cause injury and damage to the cuticle by abrasion. This would presumably facilitate subsequent germination and penetration.

The area at the base of the anal gills and around the anus previously has been found to be one of the preferred sites for penetration of *Culiseta inornata* (Williston) by *Coelomomyces psorophorae* Couch (Travland 1979). Penetration in the region around the implantation of mouthparts has also been observed with *Metarhizium anisopliae* (Metsch.) Sorok. in *Anagasta kuehniella* (Zell.) (Notini and Mathlein 1944 as cited by Veen 1966), *Schistocerca gregaria* (Forsk.) (Veen 1966), and *Hylobius pales* (Hbst.) (Schabel 1976).

Penetration through the external cuticle proper was common in larvae which were in their molting period. Penetration also occurred on exuviae. This provides further evidence of the presence of antifungal substances on the surface of intermolt larvae. These substances may be continually secreted into the epicuticle; during apolysis, the substances degrade and the old cuticle quickly looses its antifungal properties.

Vey and Fargues (1977) concluded that exuvial shedding of the invading fungus at a molt was an effective defense mechanism of *Leptinotarsa decemlineata* Say to infection by *M. anisopliae*. Sweeney (1981) also noted that early penetrations of mosquito larvae by *Culicinomyces clavisporus*

Couch, Romney and Rao are often discarded with the exuvia. The results of the present study suggest that the larvae are actually more susceptible to infection by *T. cylindrosporum* just prior to molting.

Invasion through the alimentary canal by *T. cylindrosporum* was rare and usually occurred in larvae that already had an extensive mycosis elsewhere or which were molting. However, it is possible that the fungus penetrationed through the rectum, since it was usually difficult to ascertain the precise area of penetration in this region.

Conidia often germinated within the gut during the relatively slow process of gut voiding which takes place prior to a molt. In such cases, there was generally a gradient of germinating conidia with more advanced germination and growth occurring posteriorly (Figs. 9.17, 9.18). This suggests that the movement of the food column may be important in preventing regular infections through the gut. Jaronski (1979) reported a correlation between food transportation rates within the guts and infection of mosquitoes by *Nosema algerae* Vavra and Undeen; larvae with faster rates were less susceptible.

The majority of *T. cylindrosporum* infections in field-treated larvae were initiated only after larvae were transferred to and held in the laboratory (see chapter 8) and possibly that these larvae succumbed to the fungus as a result of the physiological stress when transferred to

laboratory conditions. It is also possible that these stressed individuals ceased feeding for a period after transfer and that this lack of movement of materials through the gut provided an opportunity for *T. cylindrosporum* to germinate and establish a mycosis. Also, the molting period may be prolonged in these stressed individuals.

In the present study, penetration of the esophagus occurred within the proventriculus; penetration into the proventricular pouch was never seen. In this area, the cuticle tapers to consist of only epicuticle at the anterior end of the pouch (Richards and Richards 1971). This region may be a possible weak spot to pathogen infiltration. Conidia were not seen germinating in the anteriormost region of the midgut, i.e. in the area of the chitinous rim at the entrance to the proventricular pouch.

It is unclear if the peritrophic membrane fully envelopes the food column within the hindgut in *Ae. aegypti*. Christophers (1960) reported that the peritrophic membrane breaks up in the hindgut while Jaronski (1979) observed that it is neither broken nor torn until it reaches the rectum. In the present study fecal pellets were observed that were either completely enveloped in the peritrophic membrane or that were excreted loosely. These observations suggest that at certain times the peritrophic membrane breaks up within the hindgut. Since the peritrophic membrane did provide an initial barrier to fungal penetration within the midgut, its presence within the hindgut may also offer some protection.

Penetration of the hindgut was often observed but a relationship between the presence of the peritrophic membrane within the hindgut and host susceptibility to *T. cylindrosporum* was not ascertained.

9.4.2 Conidia vs blastoconidia

With the mosquito pathogenic hyphomycete *C. clavisporus*, the primary route of infection is through the foregut and hindgut, although conidia also randomly adhere to the external cuticle (Sweeney 1975, Sweeney *et al.* 1983). Ingested conidia adhere firmly to the gut cuticle assisted by a mucilaginous substance which encases mature conidia. Sweeney *et al.* (1983) speculated that any handling of the fungus which interferes with the conidial coating may also prevent adhesion of the conidia to the gut and impair their invasive ability even though they were viable.

Conidia of *T. cylindrosporum* are also covered in a mucilaginous coat (Fig. 9.1) but the role of this layer in pathogenesis is not known. In the present study conidia adhered only to the external cuticle. The gut walls and mouth brushes were generally devoid of conidia. Even though conidia were excreted while still viable, it is not known if the slime layer was affected.

In the present study, blastoconidia were borne primarily at ends of hyphae (Fig. 9.2). Samson and Soares (1984) noted that blastoconidia budded from short pegs on hyphae but did not mention terminal blastoconidia. Soares

(1982) noted formation of small buds at ends of hyphae penetrating the peritrophic membrane within a cadaver; he speculated these buds were incipient blastoconidia.

Soares (1979, 1982) reported that blastoconidia were very sticky and often adhered, especially at higher concentrations, to the sides of bioassay containers. He found that blastoconidia readily attached to and penetrated the integument and were more virulent than conidia. In comparison, in the present study, blastoconidia were sometimes less virulent than conidia. Gardner and Pillai (1986c) reported equal virulence between the two types of conidia, although the LT_{50}'s were shorter with blastoconidia.

Soares (1982) speculated that higher virulence of blastoconidia was due to their rapid germination. In the present study, however, blastoconidia had similar germination rates to those reported by Soares. It is possible that there are differences between laterally and terminally produced blastoconidia. There may also be differences in the adherence properties of blastoconidia produced under slightly different culture conditions. This could be a possible reason for the disparity between the results obtained by Soares (1979, 1982), Gardner and Pillai (1986c) and the present study.

9.4.3 Viability of gut-passaged conidia

Many microorganisms are able to pass apparently unchanged through the mosquito alimentary canal (Hinman 1930). Batchinsky (1927) noted that fungal spores passaged through larvae of *Culex* and *Anopheles* "developed freely on an artificial medium." In the present study I demonstrated that passage *T. cylindrosporum* conidia through the gut of *Ae. aegypti* had no affect on conidial viability. Similar results have been reported for *Metarhizium anisopliae* in other insects (i.e. Schabel 1976), although, recently Dillon and Charnley (1986) demonstrated fungitoxicity to *M. anisopliae* conidia within the gut of *Schistocerca gregaria* that was dependent on the presence of the gut bacterial flora. In *Ae. aegypti*, gut contents were purged with sterile ink prior to exposure to the inoculum, so that possible effects of gut flora on viability of *T. cylindrosporum* were excluded. However, fecal pellets, which were excreted 4 days after exposure of larvae to the inoculum under non-sterile conditions, produced an abundant growth of *T. cylindrosporum* after being placed onto PDA-SP. This indicates that the effect of gut flora was either minimal or non-existent.

9.4.4 Mortality and fungal colonization of hemocoel.

Previously I reported that between 30 to 60% of larvae exposed to *T. cylindrosporum* conidia died prior to fungal colonization of the hemocoel (chapter 7). The results of the present study indicate that most of these larvae succumbed

during their molting period.

Tolypocladium cylindrosporum frequently penetrated into the ecdysial space of these larvae. Such penetration of molting larvae also occurs in elaterids by *M. anisopliae* (Zacharuk 1973, 1974) and in *Leptinotarsa decemlineata* by *Beauveria bassiana* (Bals.) Vuill. (Fargues and Vey 1974, Vey and Fargues 1977). Such penetration frequently results in bacterial colonization of the ecdysial space which leads to host death.

Septicemia in *T. cylindrosporum* infected mosquitoes was often observed, but since detailed records were not kept, no correlations between septicemia and death during molting could be made. It is highly probable, though, that larvae without fungal colonization died as a result of septicemia.

Sweeney *et al.* (1983) observed death without fungal colonization of the hemocoel in larvae exposed to high concentrations of *C. clavisporus* conidia. Death occurred shortly after the penetration stage. It was speculated that death may be due to release of toxins by massive numbers of invading hyphae. In *C. clavisporus*, however, death without hemocoel colonization occurred shortly after larvae were exposed to conidia while in *T. cylindrosporum* this phenomenon occurred throughout the exposure period (see chapter 7).

The extent of mycosis within the larva may have a direct effect on the recycling ability of a fungus. For instance, Sweeney *et al.* (1983) found a direct correlation

between colonization of hemocoel by *C. clavisporus* and sporulation on the host cadaver.

In the present study it was found that older larvae as well as those exposed to higher concentrations of conidia had less extensive mycoses at the time of death (Figs. 9.26, 9.27). Soares (1982) also found that 2nd-instars succumb with less extensive infections of *T. cylindrosporum* than did older larvae. This phenomenon may be linked to the physiological condition of larvae. Larvae under nutritional stress may be more susceptible and die before mycosis becomes extensive. Since conidia are not digested, it can be assumed that larvae exposed to higher concentrations of conidia undergo nutritional stress as a large proportion of their diet consists of undigestible matter, i.e. conidia.

Previously, sporulation of *T. cylindrosporum* on host cadavers was observed only on floating cadavers in contact with air (Soares 1982, Samson and Soares 1984). My observation of apparent phialidic conidium development within a host cadaver demonstrates that persistence and recycling within the aquatic ecosystem may be possible. Furthermore, it indicates that large-scale production of *T. cylindrosporum* conidia may be possible in liquid fermentation. Production of conidia of entomopathogenic Hyphomycetes in liquid fermentation is rare; however, such production of conidia in submerged culture is a most desirable attribute for the mass production of a potential mycoinsecticide (Roberts and Sweeney 1982, see also chapter

4).

9.4.5 Production of club-shaped structures

Madelin *et al.* (1967) observed club-shaped structures in slide cultures of *Paecilomyces farinosus* (Holm) Brown and Smith, *B. bassiana* and *M. anisopliae* which they speculated were appressoria; structures sometimes formed in the infection process. In the present study, similar structures were observed in *T. cylindrosporum*; however, these were observed only in slide culture and, on one occasion, in hyphae adpressed to the body wall of a cadaver at the time the fungus was penetrating out of the head. Appressoria were not observed during the infection process. Soares (1982) did not observe appressoria either during the infection process or during penetration out of a cadaver. The presence of such structures in the pathogenesis of *T. cylindrosporum* is not fully understood. It is possible that such structures could be produced in confined areas such as within the sockets of the mandibles and maxillae.

9.4.6 Bioassay of *T. cylindrosporum*

In a previous study, I found that continuous exposure of mosquito larvae to conidia was not an adequate method for the bioassay of *T. cylindrosporum* (chapter 7). The results obtained in the present study provide insight into the possible sources of variability obtained in the bioassay method used; 1) ingested conidia are not digested and are

excreted viable. Thus ingested conidia move through the gut as an inert, undigestible material that would presumably have a deleterious effect on the nutrition of the larvae. 2) conidia and blastoconidia germinate within the assay cups and subsequently sporulate thus contributing to variability in dosage rates. Such germination and sporulation is presumably influenced by the microbial fauna and nutrients available within each assay container. 3) larvae are apparently more susceptible during their molting period. Therefore, the age at exposure to the inoculum may be critical. Also, presumably, the larvae under nutritional stress would have a longer molting period than well-fed larvae. 4) larvae can ingest massive doses without becoming infected once these conidia are purged. Indications are that infections are being acquired during the entire exposure period.

9.5 BIBLIOGRAPHY

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Table 9.1. Effects of gut-passage on viability of *Tolypocladium cylindrosporum* conidia in larvae of *Aedes aegypti*

	% viability±1 S.E.[1]	
Conidia	2nd instar	3rd instar
Prior to ingestion.	77±10.5	84±15.9
Ingested and excreted.	62±6.4	86±13.4
Ingested, held in gut 2.5 hr and excreted.	–	74±9.5
Held in water 2.5 hr.	80±4.9	92±5.2

[1]Mean of 5 replicates±1 Standard Error.

Table 9.2. Mean mortalities of *Aedes aegypti* larvae exposed to conidia of *Tolypocladium cylindrosporum*

Treatment	% mortality±1 S.E.[1]
Control.	9±3.9a
Conidia purged.	15±11.8a
Added to conidia on day 0.	64±10.1b
Added to conidia on day 7.	77±5.2b

[1]Means±1 Standard Error based on 5 replicates each with 10 2nd instar larvae/replicate at 25°C.
Means followed by the same letter do not differ significantly as determined by Scheffé's test at the 95% level of significance.

Table 9.3. Primary infection foci of *Tolypocladium cylindrosporum* in larvae of *Aedes aegypti* at different stages of development

	Proportion infected at each focus (%)				
	Hemocoel not colonized		Hemocoel colonized		
Infection site	Molting	Not molting	Larval molting	Pupating	Not molting
Head.	43	25	18	54	25
Head/posterior abdomen.	0	0	41	27	60
Posterior abdomen.	9	25	41	18	0
Head/thorax & posterior abdomen.	26	50	0	0	0
Other.	22[1]	0	0	0	15[1]
Sample size	23	4	17	11	12

Diagnoses made of dead cadavers serially sectioned and stained by the periodic acid method of DePalma and Young (1963).
[1]Through external cuticle of body.
[2]10% in head and thorax; 5% in thorax and posterior abdomen.

Figures 9.1 to 9.10. *Tolypocladium cylindrosporum* growth and development.

Figure 9.1. Cylindrical conidia borne aerially in slimy masses from phialides on surface of *Aedes aegypti* cadaver. (Bar=4μm)

Figure 9.2. Blastoconidia borne laterally and terminally from hyphae in submerged culture. Phase contrast. (Bar=20μm)

Figure 9.3. Phialidic conidia and blastoconidia developing within anal papilla of an infected cadaver of *Aedes aegypti*. Serial section periodic acid stained. (Bar=5μm)

Figure 9.4. Germination of blastoconidia in submerged culture incubated for 4 hr at 20°C in Sabouraud dextrose broth. Phase contrast. (Bar=20μm)

Figure 9.5. Hyphal growth resulting from a blastoconidium incubated for 24 hr at 20°C on a cellophane surface on Sabouraud dextrose agar. (Bar=20μm)

Figure 9.6. Swelling and germination of conidia incubated for 24 hr at 20°C on a cellophane surface on Sabouraud dextrose agar. (Bar=10μm)

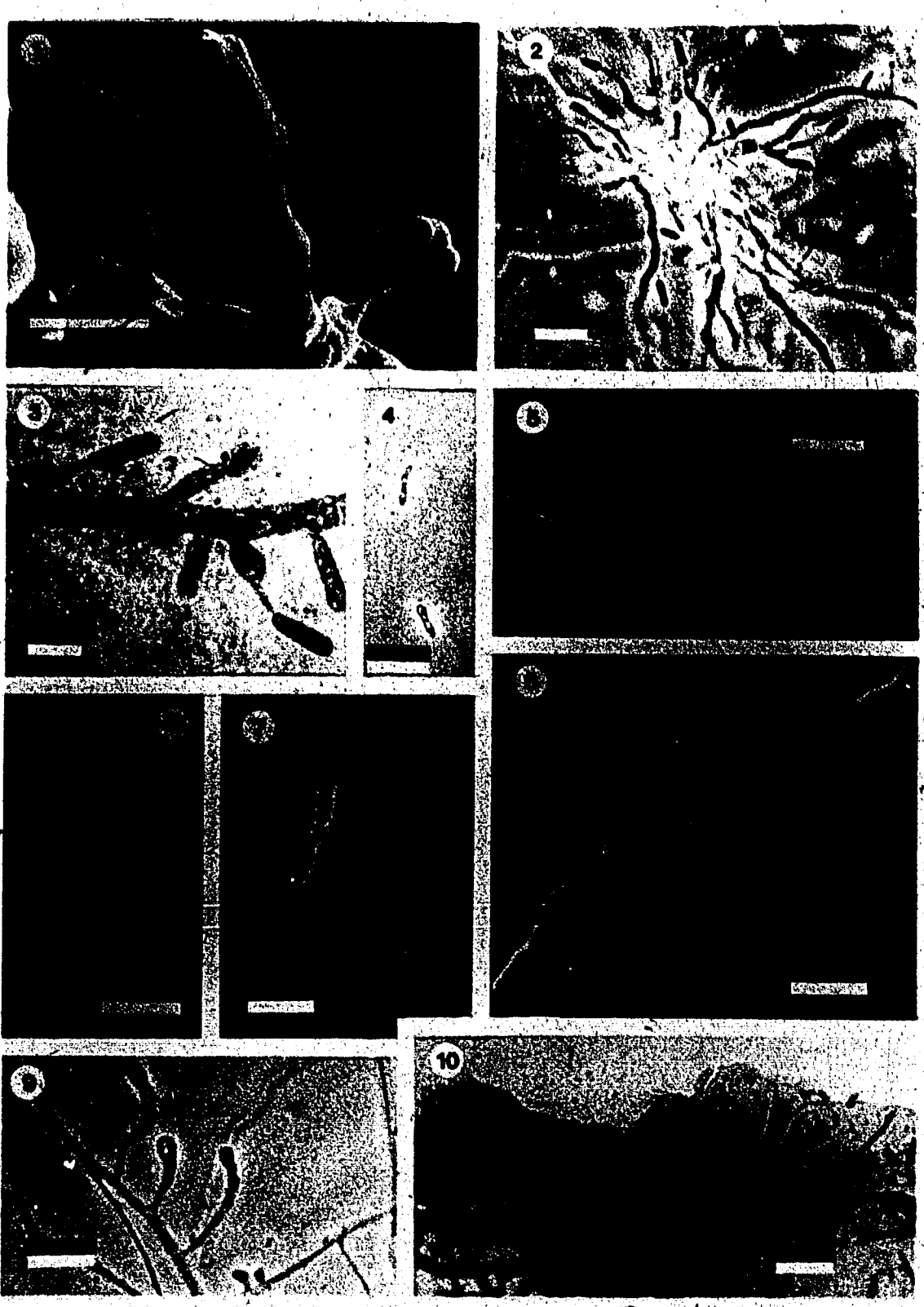
Figure 9.7. Germination of a conidium from both ends incubated for 24 hr at 20°C in Sabouraud dextrose broth. Note ungerminated conidia (arrows). Phase contrast. (Bar=20μm)

Figure 9.8. Hyphal growth resulting from a conidium incubated for 48 hr at 20°C on a cellophane surface on Sabouraud dextrose agar. Note site of original conidium (arrow). (Bar=20μm)

Figures 9.9 to 9.10. Club-shaped structures. (Bar=20μm)

Figure 9.9. Adpressed to glass surface after incubation for 7 days in slide culture at 20°C on cereal agar. Phase contrast.

Figure 9.10. Adpressed to body wall in head of an infected cadaver of *Aedes aegypti*. Serial section, periodic acid stained.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Figures 9.11 to 9.16. *Tolypocladium cylindrosporum*

Figures 9.11 to 9.12. Conidia on surface of *Aedes aegypti* larvae.

Figure 9.11. On neck 0.5 hr after placing larva into inoculum. (Bar=20μm)

Figure 9.12. On thorax 92 hr after placing larva into inoculum. (Bar=4μm)

Figures 9.13 to 9.14. Fecal pellet of *Aedes aegypti* excreted loosely 92 hr after placing larva into inoculum.

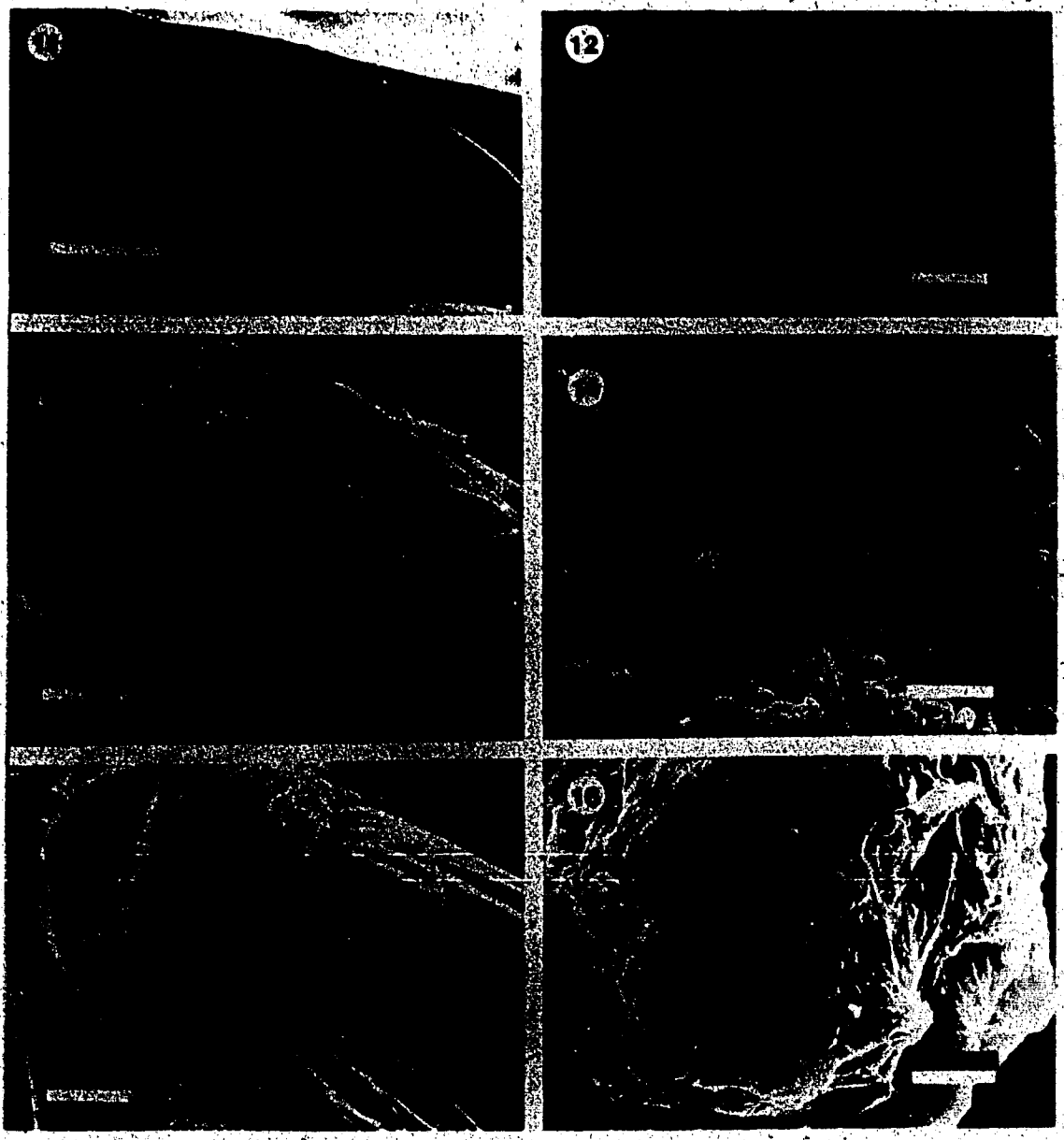
Figure 9.13. (Bar=40μm)

Figure 9.14. (Bar=4μm)

Figures 9.15 to 9.16. Fecal pellet of *Aedes aegypti* enveloped in peritrophic membrane 1 hr after placing into inoculum. Note presence of conidia beneath peritrophic membrane.

Figure 9.15. (Bar=100μm)

Figure 9.16. (Bar=10μm)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Figures 9.17 to 9.25. *Tolypocladium cylindrosporum* in *Aedes aegypti*. Periodic acid stained.

Figures 9.17 to 9.20. Germination in alimentary tract of pre-molt larvae.

Figure 9.17. In anterior portion of midgut within peritrophic membrane during voiding of midgut contents. (Bar=50μm)

Figure 9.18. In posterior portion of midgut during voiding of midgut contents. (same larva as in 9.17). (Bar=100μm)

Figure 9.19. Mycelia in esophagus within proventriculus. AM=Annular muscle. (Bar=20μm)

Figure 9.20. Germinating blastoconidium in esophagus within proventriculus. (Bar=20μm)

Figure 9.21. Head filled with mycelia. PH=Pharynx (Bar=100μm)

Figure 9.22. Posterior abdomen with ramifying mycelium. Note mycelia within fecal pellet (arrow). SA=Saddle, SI=Siphon AP=Anal Papilla (Bar=100μm)

Figure 9.23. Initial ramification of mycelia in head of a prepupa arising from penetration at base of maxilla (arrow). (Bar=100μm)

Figure 9.24. Initial penetration (arrow) at base of mandible. (Bar=20μm)

Figure 9.25. Germination of conidia in bolus within pharynx (PH). Note that mandible is filled with mycelia (arrow). (Bar=100μm)


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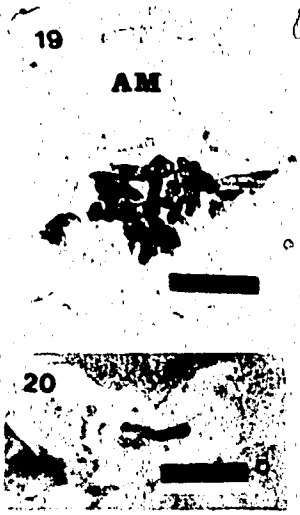
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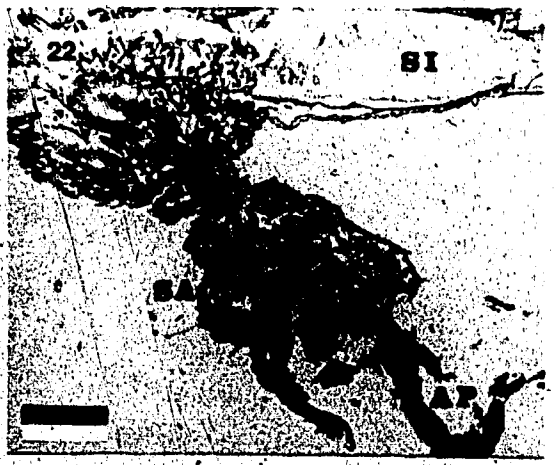
AM

20

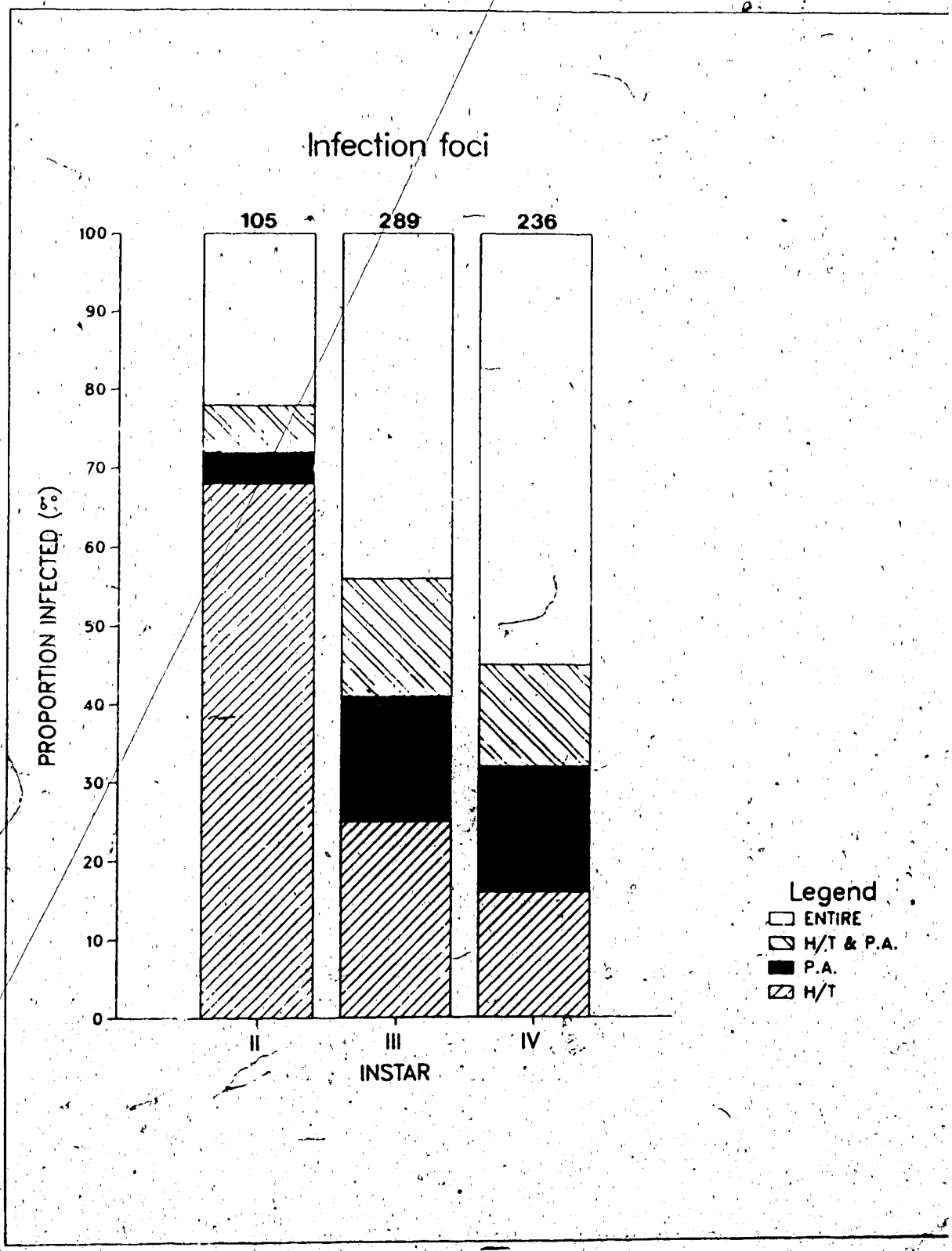

22
SI


23
PH


24


25
PH

Figure 9.26. Infection foci at time of death in relation to instar of *Aedes aegypti* larvae exposed to conidia of *Tolypocladium cylindrosporum*. Numbers above columns represent sample size. H/T=Head/Thorax, P.A.=Posterior Abdomen.


Infection foci
105
289
236
100
90
80
70
60
50
40
30
20
10
0
PROPORTION INFECTED (%)
II
III
IV
INSTAR
Legend
ENTIRE
H/T & P.A.
P.A.
H/T

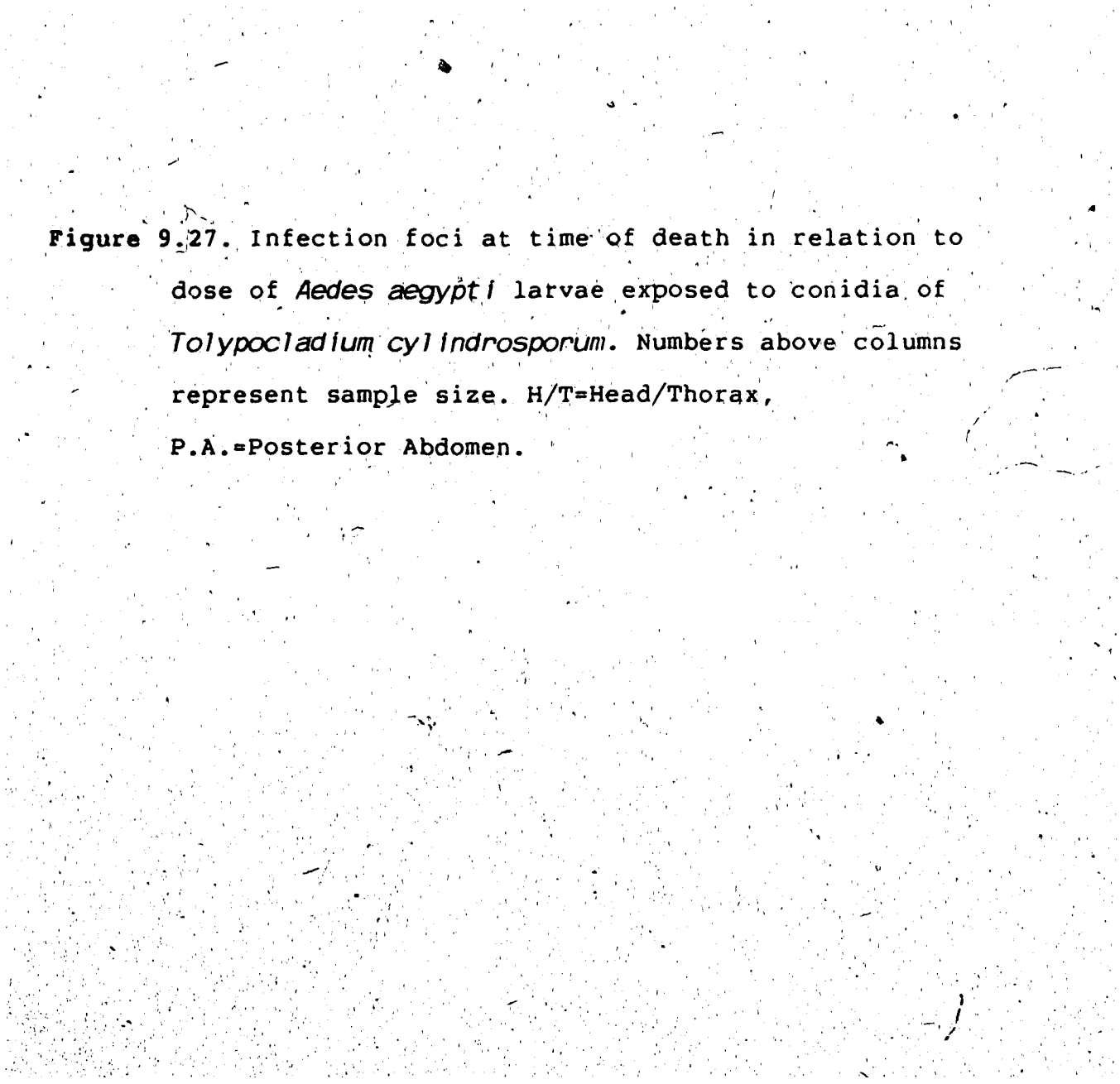
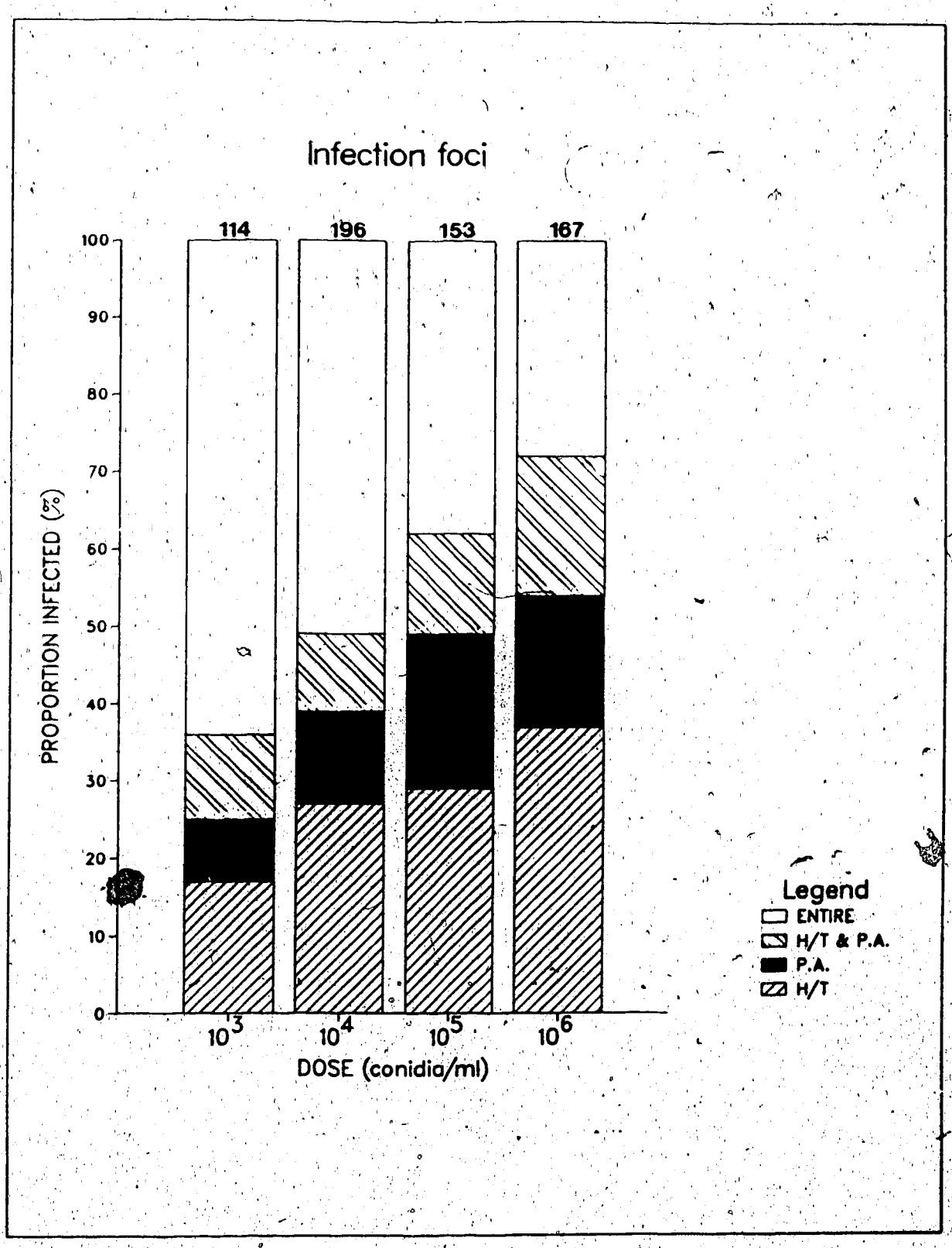


Figure 9.27. Infection foci at time of death in relation to dose of *Aedes aegypti* larvae exposed to conidia of *Tolypocladium cylindrosporum*. Numbers above columns represent sample size. H/T=Head/Thorax, P.A.=Posterior Abdomen.


Infection foci
114
196
153
167
100
90
80
70
60
50
40
30
20
10
0
PROPORTION INFECTED (%)
10^3
10^4
10^5
10^6
DOSE (conidia/ml)
Legend
ENTIRE
H/T & P.A.
P.A.
H/T

Figures 9.28 to 9.36. *Tolypocladium cylindrosporum* in *Aedes aegypti*. Serial sections periodic acid stained.

Figure 9.28. Infection through apex of antenna (arrow). (Bar=20μm)

Figures 9.29 to 9.32. Infections within proventriculus. AM=Annular muscle, BS=Blood sinus, CE=Cardial epithelium, ES=esophagus, PV=Proventriculus, RE=Reflected esophagus.

Figure 9.29. Infection through esophagus into blood sinus. (Bar=20μm)

Figure 9.30. Infection through esophagus into and at base of annular muscle. Note anterior growth of hypha (arrow) between esophagus epithelium and annular muscle. (Bar=20μm)

Figure 9.31. Hyphae growing anteriorly between the annular muscle and either the reflected esophagus or the esophagus proper (open arrows). (Bar=20μm)

Figure 9.32. Hypha emerging in a ring around the area of the muscular connective. (Bar=40μm)

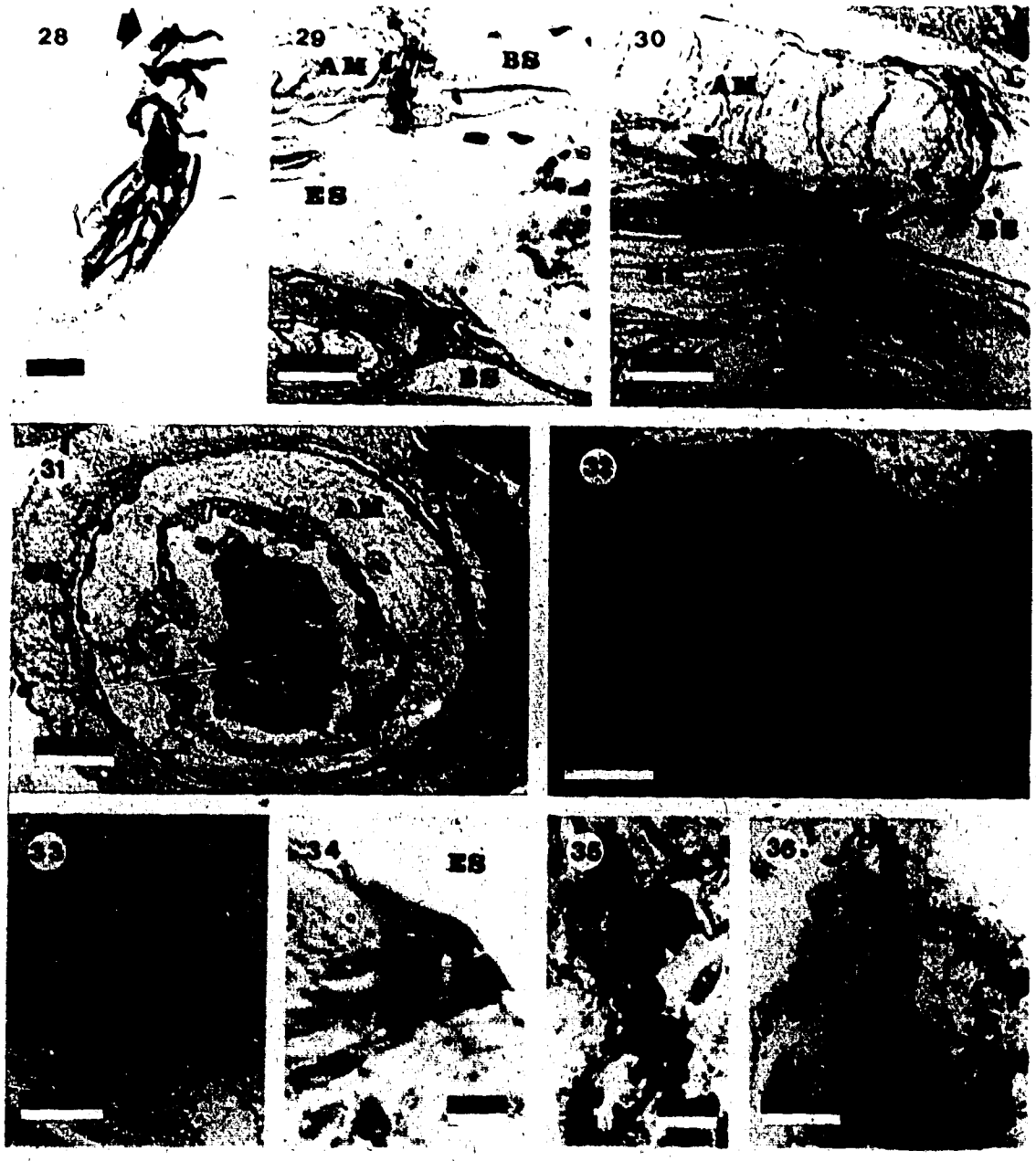
Figures 9.33 to 9.36. Melanization of invading hyphae.

Figure 9.33. Hyphae penetrating back into esophagus. (Bar=20μm)

Figure 9.34. As 9.33. (Bar=5μm)

Figure 9.35. Invading hyphae in posterior abdomen. (Bar=20μm)

Figure 9.36. As 9.35. (Bar=10μm)


28
29
AM
BS
ES
ES
30
AM
BS
31
32
33
34
ES
35
36

Figures 9.37 to 9.45. *Tolypocladium cylindrosporum* in larvae of *Aedes aegypti*. Serial sections periodic acid stained.

Figure 9.37. Mycelium within hemocoel in the region of the anus (AN). Arrow points to fecal matter. (Bar=50μm)

Figure 9.38. Invasion of the hemocoel from the rectum (RC). (Bar=50μm)

Figure 9.39. Mycelial growth in food column within peritrophic membrane in midgut. Note at top of figure peritrophic membrane sheared in sectioning. (Bar=10μm)

Figures 9.40 to 9.41. Penetration through peritrophic membrane within the midgut.

Figure 9.40. (Bar=2μm)

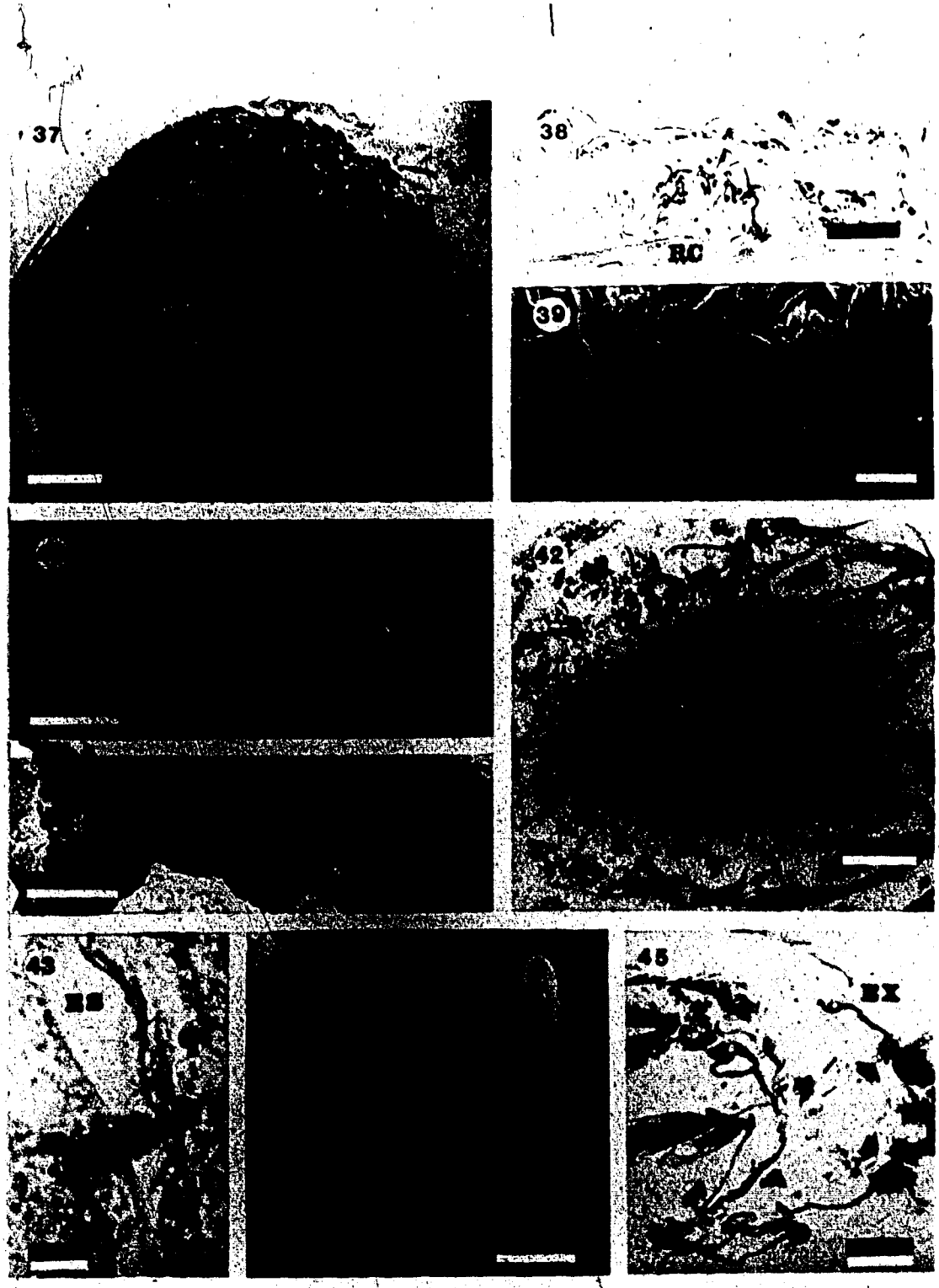
Figure 9.41. (Bar=10μm)

Figure 9.42. Penetration into midgut epithelium of a premolt larva with extensive mycosis in the head. (Bar=50μm)

Figure 9.43. Penetration into ecdysial space (ES). (Bar=5μm)

Figure 9.44. Penetrant hypha within thorax of moribund larva. (Bar=4μm)

Figure 9.45. Penetration of exuvia (EX) in the area of the mouth brushes (arrows). (Bar=100μm)


37
38
RC
39
42
43
ES
45
EX

10. General discussion, conclusions, and suggestions for future research

As far as pathogens are concerned, the aquatic flora is little known. Although numerous pathogens of mosquitoes have been reported world-wide, many occurring in epizootic situations, there have been virtually no long-term, systematic studies which provide information on the dynamics of such epizootics. Furthermore, virtually nothing is known of the role of pathogens in mosquito population dynamics. Such studies are very difficult due to the complexities of mosquito larval habitats, i.e. fluctuating populations, interactions between hosts, pathogens, parasites, and environmental conditions (Service 1981, 1985, Smith 1985). Nevertheless, if we are ever to fully exploit pathogens for mosquito control, the factors leading to epizootics in nature must be elucidated. Extensive monitoring of 10 pools over three seasons failed to detect the occurrence of epizootics in the mosquito populations under study.

The new locality and host records further demonstrate that the known geographical distribution of pathogens are merely a reflection of the geographic distribution of such studies. The extremely low prevalence and incidence of some pathogens demonstrate the difficulty of establishing such records unless long-term studies are undertaken. It is also an indication that the inoculative approach will be unsuccessful unless pathogens are genetically altered.

Before a pathogen is introduced into an ecosystem and more specifically a country, safety evaluations are first required. These studies are to determine the potential hazards of a pathogen to the ecosystem. A cost estimate for such studies in Canada is presently in the order of $Can250,000 (Morris *et al*. 1986). Limited field trials without such safety testing are in order, however, if the pathogen already occurs in the immediate locality. Therefore, locality records become extremely important as far as field studies are concerned. In the present study, I chose *Tolypocladium cylindrosporum* for field study principally because it is one of the very few mosquito pathogens known to occur in Alberta, even though isolations in Alberta are known only from soil (Bissett 1983).

Worldwide monitoring and isolation of pathogens are also required to add to the present reservoir of potentially useful microorganisms already available. The rapid commercial development of *Bacillus thuringiensis israelensis* and registration for use against mosquitoes since its first isolation is a prime example (Lacey 1985).

Monitoring and isolation of pathogens is pointless unless proper records are kept and the organisms are properly identified, catalogued, and preserved. The importance of the deposition and proper identifications of pathogen isolations cannot be over-emphasized. *Tolypocladium cylindrosporum* was mis-identified as *Beauveria tenella* (Sanders 1972, Soares *et al*. 1979, Pinnock *et al*. 1973);

another isolate was confused with *Culicinomyces* (Yu *et al.* 1980, J.S. Pillai pers. commun.). Furthermore, existing isolation records for all isolates of *T. cylindrosporum* made from mosquitoes are inadequate. Similar difficulties exist with *Culicinomyces clavisporus* (see section 3.33).

One of the major obstacles in the evaluation of a potential microbial control agent is usually the inability to produce sufficient quantities of inoculum for limited field trials. In the present study, this obstacle was overcome with *T. cylindrosporum*. However, the presence of a pathogen is not always adequate to induce an epizootic; indications were that infections did not occur in the field following mass application of *T. cylindrosporum*, although infections were apparent in larvae that were transferred to laboratory conditions.

Low temperatures may be responsible for the lack of infection mortality in the field; however, natural epizootics of *T. cylindrosporum* do occur in mosquitoes at relatively low temperatures (Weiser and Pillai 1981, Soares 1979, 1982). It is possible that, at such low temperatures, either the host or the pathogen may be favored. For instance, indications are that the movement of the food column may be important in preventing infection from within the alimentary tract. Therefore, it is possible that infections at low temperatures would be favored in species such as *Ae. australis* and *Ae. sierrensis* if these mosquitoes cease feeding at low temperatures. Conversely, species

encountered in the present study may still be actively feeding at these low temperatures.

Tolypocladium cylindrosporum is a relatively slow-acting pathogen with relatively low virulence to mosquitoes; massive doses are required to elicit a response. The host-pathogen relationships between mosquitoes and *T. cylindrosporum* are complex; multiple infection sites occur which differ according to the host's growth stage; larvae seem most susceptible during their molting period; and ingested conidia are excreted viable. This should not, however, preclude the potential of *T. cylindrosporum* as a microbial control agent. *Tolypocladium cylindrosporum* is a fungus which, under certain conditions, is able to overcome the mosquitoe's defense barriers, and cause epizootics in mosquito populations. For instance, it may be possible to use *T. cylindrosporum* in conjunction with insect growth regulators to prolong the molting period (at which time larvae seem most susceptible) or with phagodeterrents in order to inhibit feeding and allow infection *via* the gut to occur.

10.1 Suggestions for future research

In light of these findings, I would like to make the following suggestions for future research:

1) Long term and large scale field studies are needed in order to evaluate the role of pathogens in the population dynamics of mosquitoes and to isolate new species as well as

strains of pathogens which may be better suited for microbial control.

2) A better understanding of the host-pathogen interactions between mosquitoes and *T. cylindrosporum* is required. Attention should focus on the role of the conidial mucilaginous layer in pathogenesis and on the susceptibility of larvae during their molting period.

3) An adequate bioassay method for *T. cylindrosporum* needs to be developed. The possible effects of ingestion of conidia on host nutrition should be determined.

4) Further field studies with *T. cylindrosporum* are in order, preferably in simulated pools under controlled environmental conditions.

5) Attempts to select more virulent strains of *T. cylindrosporum* need to be continued. Purging infected larvae of their gut contents prior to reisolating the fungus may alleviate the difficulties encountered in the present study, i.e. probable reisolation of avirulent genotypes.

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11. Appendix 1

Table 11.1. Density and percent species composition of mosquitoes in 10 study sites in central Alberta

Site	Density[1] and species[2]	Period													
		April-May		June			July			August			Sept-Oct		
		1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984
A	Density	3.1	1.1	nm[3]	8.1	1.3	nm	13.8	d[4]	nm	d	d	nm	d	d
	Spring *Aedes*	100	100	-	-	-	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	96	100	-	100	-	-	-	-	-	-	-
	Cs. alaskaensis	-	-	-	4	-	-	-	-	-	-	-	-	-	-
B	Density	8.3	2.6	nm	18.3	4.0	nm	0.4	1.7	nm	1.0	d	d	d	d
	Spring *Aedes*	-	87	-	-	6	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	3	92	-	-	-	-	-	-	-	-	-
	Cs. alaskaensis	94	-	-	97	1	-	33	100	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	1	-	-	-	-	17	-	-	-	-
	Cs. minnesotae	-	2	-	-	-	-	-	-	-	-	-	-	-	-
	Cs. morsitans	-	12	-	-	-	-	33	-	-	83	-	-	-	
	Cx. territans	6	-	-	1	-	-	33	-	-	-	-	-	-	-
C	Density	0.9	d	0.7	1.3	d	1.2	0.4	d	2.1	1.3	d	0.4	0.1	d
	Cs. inornata	-	-	-	-	-	-	-	-	2	-	-	7	-	-
	Cs. minnesotae	-	-	-	-	-	29	-	-	7	3	-	20	-	-
	Cs. morsitans	-	-	3	-	-	2	-	-	-	-	-	-	-	-
	Cx. territans	100	-	97	100	-	69	100	-	91	97	-	72	100	-
D	Density	(2.0)	d	1.4	0.6	d	0.6	0.1	d	1.2	0.4	d	0.3	0.1	d
	Ae. vexans	-	-	-	-	-	1	-	-	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	-	-	-	-	3	-	-	-	-	-
	Cs. minnesotae	-	-	7	-	-	27	25	-	42	33	-	67	-	-
	Cs. morsitans	-	-	1	-	-	11	-	-	-	-	-	-	-	-
	Cx. territans	100	-	91	100	-	61	75	-	55	67	-	33	-	-
E	Density	1.3	0	4.5	6.1	2.5	2.1	0.3	2.3	8.3	2.4	d	2.8	0.2	d
	Spring *Aedes*	100	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	2	-	3	4	-	-	-	-	-	-	-
	Cs. alaskaensis	-	-	68	98	100	40	4	100	-	-	-	-	-	-
	Cs. inornata	-	-	10	<1	-	17	-	-	79	88	-	91	100	-
	Cs. minnesotae	-	-	5	-	-	14	14	-	8	3	-	-	-	-
	Cs. morsitans	-	-	5	-	-	9	4	-	-	1	-	-	-	-
	Cx. territans	-	-	12	-	-	17	75	-	13	9	-	9	-	-

Table 11.1. continued.

Site	Density¹ and species¹	Period													
		April-May		June			July			August			Sept-Oct		
		1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984
F	Density	2.6	1.1	2.4	14.6	2.5	0.4	3.7	1.3	6.6	8.4	0.1	2.1	1.1	d
	Spring *Aedes*	100	15	-	-	1	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	2	-	3	-	-	-	-	-	-	-	-
	Cs. alaskaensis	-	(85)	68	97	95	40	3	61	-	-	-	-	-	-
	Cs. inornata	-	-	10	1	2	17	63	4	79	99	-	91	100	-
	Cs. minnesotae	-	-	5	<1	-	14	10	15	8	<1	-	-	-	-
	Cs. morsitans	-	-	5	-	1	9	8	5	-	-	100	-	-	-
	Cx. territans	-	-	12	<1	1	17	16	15	13	<1	-	9	-	-
G	Density	(3.8)	(0.9)	nm	0.9	1.8	nm	1.0	1.9	nm	2.4	0.1	nm	0.5	0
	Ae. vexans	-	-	-	-	7	-	-	-	-	-	-	-	-	-
	Cs. alaskaensis	100	93	-	92	80	-	-	43	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	1	-	65	1	-	55	-	-	-	-
	Cs. minnesotae	-	-	-	2	3	-	3	43	-	43	-	-	-	-
	Cs. morsitans	-	-	-	-	9	-	2	6	-	-	-	-	-	-
	Cx. territans	-	-	-	6	-	-	30	6	-	2	-	-	-	-
H	Density	0	(0.4)	0.1	0.5	0.5	1.0	0.7	0.01	5.3	1.2	0	0.6	0.3	0
	Spring *Aedes*	-	2	-	-	2	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	-	11	-	-	-	-	-	-	-	-	-
	Cs. alaskaensis	-	(17)	37	33	78	-	-	-	-	-	-	-	-	-
	Cu. impatiens	-	-	12	-	-	-	-	-	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	-	14	-	-	30	6	-	33	-	-
	Cs. minnesotae	-	-	12	22	-	47	54	-	34	34	-	26	-	-
	Cs. morsitans	-	(81)	19	22	7	1	-	-	-	1	-	-	-	-
	Cx. territans	-	-	19	22	2	37	45	-	36	59	-	41	-	-
I	Density	1.5	1.1	nm	208.1	15.3	nm	48.0	d	nm	0.4	d	nm	d	d
	Spring *Aedes*	100	95	-	-	-	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	-	-	100	99	-	-	-	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	-	-	100	-	-	-	-	-	-	-
	Cs. morsitans	-	5	-	-	<1	-	-	-	-	-	-	-	-	-
	Cx. territans	-	-	-	-	<1	-	-	-	-	-	-	-	-	-

Table 17.1. continued

Site	Density[1] and species[1]	Period													
		April-May		June			July			August			Sept-Oct		
		1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984	1982	1983	1984
d	Density	1.6	1.1	nm	144.5	83.5	83.6	21.0	d	0.9	2.0	d	d	d	d
	Spring *Aedes*	100	85	-	1	-	-	-	-	-	-	-	-	-	-
	Ae. vexans	-	(15)	-	97	100	99	94	-	-	-	-	-	-	-
	Cs. alaskaensis	-	-	-	2	-	-	-	-	-	-	-	-	-	-
	Cs. inornata	-	-	-	-	-	<1	1	-	90	-	-	-	-	-
	Cs. morsitans	-	-	-	-	<1	-	-	-	-	-	-	-	-	-
	Cx. territans	-	-	-	-	(<1)	<1	5	-	10	-	-	-	-	-

[1]mean # of immatures per 350 ml dip.
[2]figures in parentheses represent results from the last collection in the time period.
[3]not monitored
[4]dry

12. Appendix 2

Table 12.1. Probit analysis of time-infection mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum*

Mosquito Species	Fungus Isolate	Temp. °C	Assay'	ST$_{50}$	95% Limits' (1x10^{3} conidia/ml)	Range'	ST$_{50}$	95% Limits' (1x10^{6} conidia/ml)	Range'
Ae. vexans	TC3	25	14		Infection <25%		6.7	6.1-7.2	3-11
		20	14		Infection <25%		6.6	6.4-6.8	6-9
			18	7.0	6.8-7.3	6-9	5.8	5.6-6.0	5-9
		15	14	15.3	14.2-16.7	12-9	10.7	10.0-11.3	8-16
		10	14		Infection <25%		13.6	12.5-14.6	7-20
Cs. inornata	TC3	20	07	12.9	11.2-14.5	7-20		nd'	
			10	11.1	10.1-11.9	5-20	7.5	6.7-8.1	5-15
			11	9.7	8.1-11.0	7-16		nd'	
		15	07	17.8	16.1-22.1	14-22		nd'	
			10	16.0	15.0-17.0	12-25	12.7	12.2-13.3	9-18
			11	17.4	17.0-17.8	13-25		nd'	
		10	10		nd'		15.5	14.4-16.5	7-25
Ae. aegypti	TC3	25	15		Infection<25%		6.6	6.2-6.9	6-9
			21	5.3	4.5-5.7	5-10	5.2	4.7-5.6	4-10
		20	15	9.7	9.3-10.2	6-15	6.8	6.4-7.1	6-12
			18		Infection <25%		6.6	6.2-7.0	4-10
			20	9.1	8.5-9.7	6-18	4.8	3.7-5.5	4-10
			21	8.8	8.4-9.1	5-16	6.9	6.6-7.2	5-12
		15	12		Infection <25%		9.1	8.1-9.8	7-19
			15	12.7	12.4-13.0	10-20	9.0	8.2-9.9	5-16
	TC4	20	16	9.1	8.6-9.7	7-15		Infection <25%	
			19	11.8	10.8-13.0	5-22		Infection <25%	
			21	8.8	8.1-9.4	5-16	6.6	6.2-6.9	5-14

Table 12.1. Continued.

Mosquito Species	Fungus Isolate	Temp. °C	Assay[1]	ST_{50}	95% Limits[4] (1x10[3] conidia/ml)	Range[5]	ST_{50}	95% Limits[4] (1x10[4] conidia/ml)	Range[5]
Ae. aegypti	TC3S6	20	20	9.6	9.1-10.2	7-14	4.3	3.4-4.6	3-11
			21	8.6	8.3-9.0	5-19	6.4	6.1-6.7	5-11
	TC3S13	20	16	8.6	8.2-9.3	7-10	7.7	7.1-8.8	5-10
			20		Infection <25%		5.7	5.3-6.1	4-12
			21	8.6	8.2-9.1	4-13	6.1	5.8-6.4	4-11
	TC3S18	20	20	8.9	8.4-9.4	6-16	7.3	7.0-7.7	4-12
			21	8.5	8.2-8.8	6-15	6.7	6.3-7.0	5-13

[1]Probit analysis of cumulative daily infection mortality as percent of total number infected and log time. Infection mortality= # individuals with diagnosed mycosis. Results of assays in which less than 25% of larvae were infected are omitted.
[2]Each assay consisted of continuous exposure of 60 2nd instar larvae per dose.
[3]ST_{50}=Survival time for 50% of total number infected.
[4]Fiducial Limits.
[5]Range between first and last infection mortality in days.
[6]Not Done.