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AN INVESTIGATION OF THE PATHOGENIC RESPONSES OF AEDES TRISERIATUS(SAY) (DIPTERA:CULICIDAE) TO INFECTION BY LEPTOLEGNIA SP.(OOMYCETES:SAPROLEGNIALES)

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Entomology

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Major professor

Date August 5, 1987

O-12771

AN INVESTIGATION OF THE PATHOGENIC RESPONSES OF *AEDES TRISERIATUS* (SAY) (DIPTERA : CULICIDAE) TO INFECTION BY *LEPTOLEGNIA* SP. (OOMYCETES : SAPROLEGNIALES)

By

Bassey J. Akpan Eyo

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

1987

ABSTRACT

AN INVESTIGATION OF THE PATHOGENIC RESPONSES OF *AEDES TRISERIATUS* (SAY) (DIPTERA : CULICIDAE) TO INFECTION BY *LEPTOLEGNIA SP.* (OOMYCETES : SAPROLEGNIALES)

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The larvae of the tree hole mosquito *Aedes triseriatus* (Say) were inoculated with the mosquito pathogen, *Leptolegnia sp.*, established on half split hemp seed. Mode of infection, pathological and histopathological responses, viability, pathogenicity and orientation were investigated.

Infectivity tests showed that zoospores released from freshly prepared hempseed inoculum induced 78% to 96% larval mortality within 24 hours, and 100% within 48 to 72 hours, with LT_{50} of 11.5 hours. When used on repeated tests, this inoculum persisted for 27 days killing five batches of mosquito larvae at the rate of 99% per batch within 72 hours. In orientation tests, motile zoospores preferred larval homogenate to agar medium.

Viability and pathogenicity was higher in inoculum stored at $5^{o}C$ than one stored at $25^{o}C$ for 12 months. On infectivity tests, inoculum stored at $5^{o}C$ induced 91% larval mortality within 72 hours. Reduced viability in inoculum stored at $25^{o}C$ resulted in declining larval mortality. *Leptolegnia sp.* cultured on agar slants of

various media remained viable after 24 months of storage at 25°C.

Microscopic examination of larvae after inoculation revealed that infection occurred primarily through ingestion and secondarily through anal and spiracular openings. Symptoms of infection include excessive grooming, gaping of mouthparts, palpitation, change in color and position, cessation of feeding, reduction in wiggling, eventual sluggishness and death. The presence of zoospores in the hemocoel or target organ is a definitive early diagnosis of infection. Larval susceptibility to infection remained the same with fed and unfed larvae.

Target organs for infection were primarily the mouthparts, esophagus, crop, stomodael membrane, midgut, trachea and anal papillae, and secondarily, gastric caecum, rectum, and hindgut. Infected larvae died from: (a) cytological damage to tissues in infected organs,(b) suffocation from encysted spores blocking the tracheae (c) destruction of hemocytes and fat bodies. Infection often aroused melanization of encysted zoospores and sporangia within larval hemocoel.

DEDICATION

TO THE MEMORY OF MY HUSBAND
AMAJAK EMMANUEL EYO

"The World's an inn, and death the journey's end."

Dryden

But for God's own children, there's yet a higher life,
The one that you've entered, to prepare a place for us,
I'll see you there smiling, when my days are done.
But before then my darling, permit me to recall,
Your thoughtful understanding, your deep belief in me,
Your support that got me started, your prayers that sustained.
In death as in life, your encouraging words I hear,"
You can do it, honey, the kids and I are fine."
And so I kept on pushing, 'til this stage I find,
The task is now accomplished, and I need you to share.
Replace these empty feelings with your cheery smiles of care.
For there's life to hope for when earthly hopes are gone,
And let me thank you, BUDDY, for the supreme gift of LOVE.

Bassey J.A. Eyo

"We loved with a love that was more than love"

Poe

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to my major advisor, Dr. Harold D. Newson for his continued support, encouragement, understanding, and guidance throughout the course of my Ph.D. program. The kindness and understanding of his wife, Mrs. Beverly Newson will always be appreciated. Thank you both for ALL you have done for me and my family.

I also want to express my gratitude to other members of my committee, Dr. E.S. Beneke, Dr. K. Klomparens, Dr. F. Stehr and Dr. S.G. Wellso for their invaluable support, suggestions and guidance. Our former chairman, Dr. J. Bath deserves special gratitude for giving me the opportunity, support, and encouragement throughout this challenging program. The support, understanding and compassion of his faculty, staff and students kept me "sane" at times of great loss and enhanced the continuation of this program.

I want to express my sincere appreciation to Dr. E.S. Beneke for taking me into his laboratory and treating me as one of his own students. His patience, understanding, encouragement, support, and guidance will be remembered and appreciated. Thank you also for the helpful support to my family. Appreciation is also extended to Dr. A.L. Rogers for accepting me in the laboratory and providing many scientific insights that soothed my hours of research.

My special gratitude goes to Dr. Klomparens for her patience, suggestions and encouragement. Thanks also to Dr. S. Flegler and Dr. J. Heckman and other staff and students past and present at the Center for Electron Optics.

I am grateful to Dr. J. Miller and Dr. S.W. Wellso for providing the spaces for my insectory, and to Dr. G. Craig of the University of Notre Dame for supplying the eggs with which this research was initiated.

I am also grateful for the friendship of Massummeh Ahmadizadeh, Diann Jordan, Isabella Fernandez, Ramon Sandin, Jan Morris and Donna Duberg with whom I have enjoyed many hours of shared experiences. Special appreciation to Donna for "boosting" my energy through many late nights. Thanks also go to Kathryn Smith, James Robert, James Kidder William Morgan, Peter Carrington, John Hayden, Kenneth Dimoff, Linda Carroll and the students and staff past and present in the Medical Mycology and Medical Entomology laboratories.

Sincere appreciation is extended to my brothers Ben and Jacob Akpan for their constant support and encouragement and for keeping my parents' dream of academic achievements in me alive. Last and not the least, I want to thank my children Unwana, Ikemesit and Senyene for "putting mom through school" with their love, tenacity, endurance, understanding and support. You have indeed taught me the joys of motherhood. You are truely the pride of this accomplishment.

Two roads diverged in a wood, and
I took the one less traveled by
And that has made all the difference.

Robert Frost

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INTRODUCTION

The use of fungi to control insects of medical and veterinary importance was advocated by Hagen (1879) working with the housefly fungus, but that idea did not receive wide consideration until the advent of biological control concepts in recent years. Increased problems of mosquito resistance to conventional chemical insecticides, non-selectivity of these insecticides and their tendency to contaminate the environment, in addition to the high cost of application, have fostered interest in the use of natural enemies as biological control agents. Interest in research on insect mycosis as a pest suppression measure for mosquitoes was spurred by publications from Jenkins (1964), Laird (1970, 1971), Chapman et al. (1970, 1971, 1972) and Chapman (1974).

In 1979, the World Health Organization (WHO) listed Leptolegnia among six fungi earmarked for research as biological control agents, (Davidson and Sweeney 1983). Since then, a number of research efforts have been made not only to determine the species of pathogenic Leptolegnia (Seymour 1984) and their nutritional requirements (Nolan 1983) but also their host range in mosquitoes and other

larval Diptera (McInnis and Zattau 1982, McInnis et al. 1985). All work so far reported about pathogenic Leptolegnia has been in the laboratory, and all laboratory research so far conducted has met with success. There has been no reported field trial for Leptolegnia sp.. Before any field trial could be deemed successful, certain basic information regarding the host, the pathogen, their ecologic systems and the types of interaction that exist between them must be obtained. McCray et al. (1973) stated that before any pathogen is selected as a candidate for field experimentation against mosquitoes, basic information regarding infectivity, portal of entry and exit, parasite-host relationship, tissues or organs primarily attacked, storage, production, environmental factors, among others must be compiled.

Successful infectivity studies in the laboratory have so far enlisted Leptolegnia sp. as a mosquito pathogen. If this known pathogen is ever going to be employed as a biological control agent more research is needed to determine, among other factors, its interactive relationship with the host with which it had co-evolved, the mode of infection, the mode of action of the pathogen once inside or on the body of the host, the pathological processes in an infected host, physical and biological

factors that may affect infection, persistence and pathogenicity, and the means of storing the infective unit or embodiment of it. Since both pathogen and host had co-evolved in a given ecosystem, a biological control program that seeks to utilize insect mycosis as a control measure should be embarked upon with a clear understanding of the mechanism of penetration of the fungal pathogen into the the body of the mosquito host and the reaction of the host to that infection and invasion by the pathogen.

Knowledge of host interaction will enhance an understanding of any defense mechanism developed by the host when subjected to stress by epizootics of this fungus. Knowledge of penetration or mode of infection will help in formulation of this fungal pathogen as a larvicide, and in the development of appropriate application technique(s). Understanding the developmental process of the pathogen within the host, coupled with knowledge of the conditions under which infection and invasion occur, will have implications on the etiology and epizootiology of the disease. Knowledge of storage temperatures and storage periods will help in mass production, packaging and distribution or transportation of this fungal pathogen. Knowledge of persistence and orientation will shed some light on the behavior of the pathogen, its host searching

ability, its longevity, and its overall ability to control the intended mosquito pest population. In consideration of all these factors, the research project here reported was undertaken to provide baseline information that will contribute to the successful development of Leptolegnia sp. as a larvicide, or the incorporation of it in an integrated mosquito management program.

This dissertation presents:

A. The bioassay study which details a technique under which the infective unit, the zoospore, could be applied to the host population.

B. Study on persistence, which demonstrates that this pathogen is capable of persisting in a given environment to infect more than one population of the mosquito larvae.

C. Study on orientation which describes the ability of a zoospore to orientate itself towards a mosquito larva as a preferred medium upon which to encyst.

D. Study on mode of infection, a carefully sequenced documentary of the paths of zoospores that have infected the mosquito larvae, development of the

infection process, and the response of the larval host to infection by the fungal zoospores.

E. Study on starvation which seeks to determine the effect of food availability and scarcity on the susceptibility of the larvae to infection by the pathogen.

F. Investigation on storage conditions, substantiates the effect of temperature, months in storage and growth media on the viability and pathogenicity of Leptolegnia sp. infecting the larvae of Aedes triseriatus (Say).

G. Pathology and histopathological studies which document the targeted organs for infection, describes the damage done to the organs and the tissues infected, and also presents symptoms for diagnosis of leptolegnial infection in Aedes triseriatus larvae.

LITERATURE REVIEW

A. The Host: *Aedes triseriatus* (Say).

1. Medical Importance

Aedes triseriatus, (Diptera: Culicidae), the most widely distributed treehole-breeding mosquito in North America, has been implicated as a vector of a number of pathogenic microbial organisms to man and animals. Studies in recent years have identified this species as a known vector of the LaCrosse (LAC) strain of California encephalitis virus (Masterson et al. 1971, Sudia et al. 1971, Thompson et al. 1972 and Watts et al. 1972, 1973a). DeFoliart (1982) observed that almost a hundred cases of LAC virus encephalitis were reported annually from the eastern two-thirds of the United States. Cases of LAC virus in Ohio and Minnesota are linked to *Ae. triseriatus* breeding in pockets of water around residential and farm areas (DeFoliart and Liseitza 1980, DeFoliart 1983).

All stages of *Ae. triseriatus* exhibit some form of vector potential. The eggs serve as a vehicle for vertical (transovarial) transmission and also as a mechanism for overwintering of the LAC virus (Watts et al. 1973b, 1974).

The virus has also been isolated from the larvae (Pantuwatana et al. 1972). The adult females perpetuate the occurrence of LAC virus by engaging in vertical (transovarial) transmission via their eggs, and horizontal transmission through infective bites on chipmunks and tree squirrels which serve as amplifiers for the virus being transmitted from infected to uninfected female mosquitoes (Watts et al. 1972, 1973a, 1973b, DeFoliart 1983). Venereal transmission of this virus between females and males of Ae. triseriatus has been reported (Thompson and Beaty 1977, 1978).

Vectorial efficiency of Ae. triseriatus has not only been limited to LAC virus. This mosquito species has also been suspected as a vector for eastern equine encephalomyelitis (Davis 1940, Chamberlain and Sudia 1961, Whitefield et al. 1971). Further reports have incriminated Ae. triseriatus in transmitting microfilariae that cause dog heartworm disease, which has become an increasing problem in Michigan (Lewandowski 1977, Rogers 1980). Similar findings have been documented in Massachusetts (Phillips 1939), Texas (Keegan et al. 1968), and Mississippi (Intermill 1973). In addition to transmitting diseases to mammals, Ae. triseriatus has also exhibited a vector potential for bird malaria (Huff 1932).

2. Distribution and Breeding Sites

The potentiality of this vector to spread any of the diseases cited could be estimated when one considers its vast distribution and unusual breeding habits. Horsfall (1955) stated that, in the southern United States, this mosquito species became a pest in proportion to the availability of its breeding sites. Ecologists have reported that the range of this mosquito species extends from the deciduous forests of eastern United States and southern Canada (Zavortink 1972), through most of the states east of the Rocky Mountains (Hardwood and James 1979), southward to the Florida Keys and Texas, and westward to Utah and Nebraska (Zaim 1978). Its preferred habitats are rot holes of deciduous trees from subtropical Florida about 25^{o}N through the temperate zones of the mid-Atlantic and midwest states latitude 40-42^{o}N to central Canada (Bradshaw and Holzapfel 1985).

The natural breeding sites of Ae. triseriatus are tree holes that contain water. DeFoliart (1982) found that in western Wisconsin, large gallery forests are the primary centers for production and dispersion of this species. Petersen and Chapman (1969) found that Ae. triseriatus breeds readily in treeholes, stumpholes and ground cavities with low salinity. In Arkansas, Horsfall (1955) reported

high larval population in all kinds of containers that were shaded and sheltered from the wind, in rot holes in thickets of sweetgum trees along flood plains of tributary streams and in choked gutters of houses. Field studies have revealed increasing expansion in breeding sites of Ae. triseriatus from natural treeholes, water-filled rot holes in trees, and ground cavities to artificial containers such as tins, cans, bird baths, discarded tires and rain gutters (Williams 1962, Wilton 1968, Beier and Trpis 1981 and DeMaio et al. 1981).

Numerous studies have indicated that larvae and pupae exist in these diverse water containers where oviposition occurred, while the adults disperse to wooded areas during the day (Loor and DeFoliart 1969, 1970, Furlow and Young 1970, Sinsko and Grimstad 1977, Nasci 1982, Novak and Peloquin 1981), or to houses (Horsfall, 1955). Lenehan (1983) reported that as Midwestern forests are being replaced by shopping malls, subdivisions and other real estate developments, Ae. triseriatus is adapting more readily to breeding in scrap tires and holes in the trees found in parks and recreational areas. Successful adaptation to tire breeding is documented in the finding that strains of Ae. triseriatus that breed in tires are

larger in size, live longer and emerge much earlier than strains that breed in treeholes (Haramis 1983).

3. Laboratory Colonies and Control Measures

The recognition that Ae. triseriatus is the principal vector of LAC virus in the Great Lakes states has stimulated a lot of research on this species. A large proportion of this research has been facilitated by establishment of laboratory colonies. Repass (1952) established a laboratory colony of Ae. triseriatus, rearing the adults in 2 x 2 x 3 inch cages in a room with a temperature of 77°-79°F and humidity of 75-80%. The larvae were fed with pulverized dog biscuits. Gerberg (1970) detailed the procedures for collecting and storing the eggs, rearing and maintaining larvae, pupae and adults under laboratory conditions. Several modifications have been made on the Repass (1952) and Gerberg (1970) techniques (Hayes and Morlan 1957, Rogers 1980, Fortin and Slocombe 1981, Novak and Peloquin 1981) to enhance successful establishment of large populations of different stages of Ae. triseriatus in the laboratory.

The control of Ae. triseriatus by the conventional chemical methods has been very difficult because sprayable chemicals do not reach the larvae in treeholes or the

adults in wooded forests. Craig (1983) elaborated on the inadequate and difficult control methods for this species. He stated that control measures have been handicapped by unsatisfactory population monitoring methods due to their breeding habits, species complex, genetic variability, and also by a lack of satisfactory ways of disposing of steel-belted tires. In respect to the difficulties cited, various approaches to control are being investigated. The use of a plant-derived chemical oviposition attractant, 4-methylcyclohexanol, has been successful in the laboratory but not yet tried in the field (Bentley et al. 1982). In western Wisconsin, where LAC virus is endemic and Ae. triseriatus breeds in large forests, efforts have been made to reduce breeding sites by filling tree holes and properly discarding man made containers (Garry and DeFoliart 1975, Scholl et al. 1979, DeFoliart 1982).

Investigations on the use of biological control agents to decimate the population of Ae. triseriatus have yielded some success in the laboratory, while results on field trials are yet undetermined. DeMaio et al. (1981) found that Bacillus thuringiensis var. israelensis worked against Ae. triseriatus in treeholes and tires but loses its toxicity within 3-5 days. A nematode, Romanomermis culicivorax infected Ae. triseriatus, but the host produces

an immune response against the pathogen after a brief period of infection (Poinar et al. 1979). In preliminary laboratory studies, Ae. triseriatus has been found to be susceptible to infection by a nuclear polyhedrosis virus (NPV) and aquatic Oomycetes fungi, Lagenidium giganteum (Federici and Anthony 1972a, Federici and Lowe 1972b) and Leptolegnia sp. (McInnis et al. 1985).

B. The Pathogen: Leptolegnia sp.

1. Historical Background

Sparrow (1960) described the genus Leptolegnia deBary (Oomycetes:Saprolegniales) and cited one undescribed species, "Leptolegnia sp. Vallin" as a suspected agent for an epidemic among copepods. Different isolates of parasitic Leptolegnia sp. have since been described and reported as potential pathogens of mosquitoes. Seymour (1976) described an isolate of Leptolegnia sp. from parasitized mosquito larvae in central Ohio. Further attempt was made by Seymour (1978) to correlate the Ohio isolate with the Clemson, South Carolina isolate. McInnis Jr. and Zattau (1982), McInnis Jr. et al. (1985) have investigated the biological control potentials of the South Carolina isolate of Leptolegnia sp.. Irrespective of all the descriptions and investigations the species of this

parasitic Leptolegnia remained unknown until Seymour (1984) embarked on a taxonomic description and illustration of a parasitic Leptolegnia sp. which he described as a new species and gave the scientific name of Leptolegnia chapmanii. He collaborated previous work on Leptolegnia sp. and also recognized Chapman for identifying Leptolegnia sp. from parasitized Aedes triseriatus larvae in Lake Charles, Louisiana (Seymour 1984).

The Leptolegnia sp. used in my investigation was obtained from American Type Culture Collection (ATCC) identified as ATCC^{R} 36191, L.G. Willoughby 773, maintained on medium 307 20C. To ascertain if the Leptolegnia sp. used in this study, fits the description given by Seymour (1984) and warrants the recognition of the species name for my purpose, a letter was sent to Dr. S. Jong, head of the Mycological Department at ATCC.

The following is an excerpt from that correspondence. From my letter to Dr. Jong:

> I received, batch number 36191, a fungus called Leptolegnia sp. from your agency. Literature materials had been referring to this pathogen as having an unknown species. There is an article in Mycologia 76(4), pp. 670-674, in which Dr. Roland L. Seymour describes a fungal pathogen whose species has hitherto been unnamed; and he named this species chapmanii. I am writing here to ascertain from you whether the newly described Leptolegnia chapmanii is the same as Leptolegnia sp. I obtained from you and if the name has been so accepted and substituted in your agency.

From Dr. Jong's reply:

> We have been studying our stock culture of Leptolegnia sp. ATCC 36191. We have now confirmed that ATCC 36191 is not Leptolegnia chapmanii which was established as a new species by Dr. R.L. Seymour in Mycologia 76 (4):670-674, 1984.

All references to the pathogen used in this investigation is made to Leptolegnia sp. ATCC^R 36191.

2. Biology and Life Cycle

An unnamed species of Leptolegnia, (ATCC^R 36191), (Oomycetes: Saprolegniales), is an aquatic fungus with a filamentous, eucarpic, non-septate thallus. The genus is described as having hyphae that are long, slender, delicate, sparingly branched and have a wall composed largely of cellulose and B-glucans (Couch 1924, Sparrow 1960, Alexopoulos and Mims 1979). Long, straight sporangia are formed from the filamentous, undifferentiated, vegetative hyphae. Spores, formed in a single row within the sporangia (zoosporangia) are elongate on discharge, form an oval shape and swim immediately after discharge from a pore at the tip of the sporangia (Sparrow 1960, Seymour 1978, Alexopoulos and Mims 1979).

These swimming spores (zoospores) are dimorphic, diplanetic, biflagellated, and have anteriorly directed tinsel and posteriorly directed whiplash flagella. Upon

emergence from zoosporangia, the motile primary zoospores become pear-shaped with apical flagella, and swim for a period of time before encysting to form a big round primary spore. After a few minutes, the encysted primary spores emerge as kidney-shaped secondary motile zoospores with laterally located flagella, and swim for a longer period before encysting into another round-shaped spore smaller in size than the encysted primary spores (Alexopoulos and Mims 1979, Sparrow 1960).

This fungus exhibits both asexual and sexual reproductive patterns. Asexual reproduction involves encystment and germination of zoospores released from cylindrical, terminally located zoosporangia. Sexual reproduction in known species of Oomycetes fungi involves gametangial contact preceded by meiosis, which occurs in the gametangia, and is followed by formation of zygotes (oospores). The oogonial walls of some species of Leptolegnia are unpitted, densely ornamented and stalked. The male gametangia includes laterally appressed antheridia (Sparrow 1960, Alexopoulos 1966, Alexopoulos and Mims 1979, Moore-Landecker 1982, Seymour 1984).

3. Habitat and Laboratory Culture

Leptolegnia sp. is an uncommon Oomycetes fungus that inhabits freshwater where it can be isolated from various organic substrates, and occasionally from moist soils and marine invertebrates (Coker 1909, Couch 1924, Seymour 1978, McInnis and Zattau 1982, Nolan 1983).

In the laboratory, Leptolegnia sp. has been cultured on a variety of media. Nolan (1983) found that a species of Leptolegnia grows readily on an agar plate of Emerson's yeast extract, soluble-starch (EYPSS) medium, but zoospore production was increased when transferred to V-8 juice agar. McInnis and Zattau (1982) cultured Leptolegnia sp. on Difco corn meal agar supplemented with dextrose and peptone (CMDP) but found that increased zoospore production was achieved when agar blocks with growing mycelia were transferred to deionized, distilled water. Seymour (1978) maintained the culture of Leptolegnia sp. on Difco corn meal agar (CM) but initiated and increased zoospore production by transferring agar blocks into deionized water and letting the fungus grow on split hemp seed added to the water. McInnis et al. (1985) maintained broth culture of Leptolegnia sp. by homogenizing the fungus in CMDP medium, placing it in a broth of peptone-yeast extract-glucose

(PYG) and incubating this homogenate on a rotary shaker for 4 days at 25°C.

4. Pathogenic Potentials

Leptolegnia is one of the six genera of fungi currently being evaluated as potential mosquito larvicides (Davidson and Sweeney 1983, WHO 1985). Leptolegnia sp. has been reported as a facultative parasite, virulent against Culex pipiens quinquefasciatus, Anopheles quadrimaculatus, Aedes aegypti, and Anopheles albimanus (McInnis and Zattau 1982). Other species of Leptolegnia have been reported to infect a freshwater nematode Neomesomermis flumeralis (Nolan 1983). Early isolates of Leptolegnia from fresh water habitats were found to be infective for gnats, flies, wasps, mosquitoes, and spiders (Coker 1909), termites and mushroom grubs (Couch 1924). Coker (1923) isolated Leptolegnia and other Saprolegniaceae growing as saprophytes on a number of dead organisms and cited that the Leptolegnia species exhibited potential as a parasite. Although no successful field infection has yet been documented, Seymour (1976) isolated Leptolegnia sp. from parasitized Aedes triseriatus.

In a host range study, McInnis et al. (1985) found that Leptolegnia sp. demonstrated a high degree of

specificity in parasitizing larvae of Culex pipiens, Culex salinarius, Culiseta inornata, Aedes taeniorhynchus, and Aedes triseriatus. Leptolegnia sp. has been referred to as a "mosquito pathogen" because of its ability to induce 70 -100% mortality in first to third larval instars of nine species of mosquitoes under laboratory conditions (McInnis and Zattau 1982, McInnis et al. 1985). In summarizing research on the biocontrol of vectors of diseases, Burges et al. (1981) noted that Leptolegnia sp. has a broad mosquito host-range which includes species of Culex, Aedes, and Anopheles, and that a zoospore concentration of 10^3/ml would kill 100% of larvae under laboratory conditions.

5. Preparation of Inoculum

The infective stage of this pathogen is the biflagellated primary and secondary zoospores. Two different methods of infecting the hosts with zoosporous units have been described (McInnis and Zattau 1982, McInnis et al. 1985). The earlier method involved placing 0.5 cm disks cut from edges of a culture growing on CMDP agar in crystallizing bowls and allowing sporulation to occur before adding the mosquito larvae. In later procedures, mycelia collected from PYG agar broth were washed, placed in infection bowls and allowed to sporulate before adding

the host species. The use of hemp seed to secure units of infection was reported by McCray (1973) who placed hemp seeds on the surface of agar culture of Lagenidium culicidum for 7 days. After 7 days, these hemp seeds were transferred to 100 ml. of distilled water into which 100 larvae were introduced. The larvae that became infected from the hemp seed units were in turn used for inoculating healthy larvae.

C. Investigations on Ae. triseriatus Infected with Leptolegnia sp.

References on interactions between Ae. triseriatus and Leptolegnia sp. are limited to the isolation of Leptolegnia sp. from parasitized Ae. triseriatus (Seymour 1976) and the susceptibility of Ae. triseriatus to infection by Leptolegnia sp. during host range studies (McInnis et al. 1985). There has been no documented investigation on the pathology or any form of interaction between this host and this pathogen, but noteworthy studies have been done on other pathogenic fungi interacting with other insect larvae.

1. Bioassay and Persistence of *Leptolegnia* sp. Infecting *Ae. triseriatus* Larvae

Studies on pathogenic activities of *Lagenidium giganteum*, an aquatic, facultative parasite of mosquito larvae, indicated that infection was accomplished within 24 to 72 hours after exposure to the host (Couch and Romney 1973, Umphlett and Huang 1972, Washino 1981, Merriam and Axtell 1982). In demonstrating susceptibility of *Aedes aegypti* to infection by *Leptolegnia* sp., McInnis and Zattau (1982) noted that 100% mortality occurred among first and second instar larvae within 24 hours while less than 40% of third and fourth instar larvae were infected after 72 hours of exposure. Sweeney et al. (1983) assayed larval mortality of *Culex annulirostris* parasitized by *Culicinomyces clavisporus* post-exposure time of 1, 24, and 48 hours. Determination of lethal time for fifty percent of a given population (LT_{50}) is a known practice when testing the susceptibility of target insect pest to chemical insecticides per set time. Soares and Pinnock (1984) used LT_{50} in days to analyze the effect of temperature on infectivity of the mosquito pathogen *Tolypocladium cylindrosporum*.

Some Oomycetes parasitic on mosquito larvae have demonstrated persistent infection of their hosts.

Lagenidium giganteum remained viable and persistently virulent against several species of mosquito larvae in seepage ditches associated with rice fields and in irrigated pastures in California (Washino 1981, McCray et al. 1973, Christensen et al. 1977). L. giganteum also was a persistent pathogen in natural freshwater mosquito breeding sites in a flooded woodland in North Carolina (Jaronski and Axtell 1983) and in flooded depressions and a black gum swamp in Louisiana (Glenn and Chapman 1978).

2. Orientation of Zoospores of Leptolegnia sp. Towards Ae. triseriatus Larvae

One of the problems envisaged in the use of microscopic organisms as biological control agents is the ability of such an agent to actively search for, identify and infect its host. Heimpel (1972) cited that an effective insect pathogen must demonstrate the following characteristics: (a) efficiency (b) specificity (c) ease of production (d) storage and (e) formulation. McInnis et al. (1985) alluded to the fact that Leptolegnia sp. demonstrated a high degree of specificity towards mosquito larvae and did not infect nontarget organisms that he tested. No study has been done on the ability of the infective leptolegnial zoospores to find the target host.

It has been suggested that parasitic fungi find their hosts by chemotaxis, chemotropism, chemokinesis or a combination of chemotaxis and chemotropism, but the mechanism of such orientations has not been elucidated. Zoospores of Phytophthora, Pythium and related species of Oomycetes reach their host plants by chemotaxis, adhere to host surface and chemotropically develop a germ-tube by which the host is penetrated (Zentmyer 1970, Hickman 1970, Young et al. 1979, Carlile 1975). A wide range of compounds, present in hosts, were found to elicit chemotactic and chemotropic responses, adhesion and encystment in plant pathogenic zoospores (Cameron and Carlile 1978, Khew and Zentmyer 1973, Sing and Bartnicki-Garcia 1972, Carlile 1980). McInnis (1971) and Nolan (1983) reported that the zoospores of the Oomycetes, L. giganteum and Leptolegnia sp., respectively, parasitize their insect hosts to acquire sterol and other organic chemicals needed for their nutrition; but it has not been documented that any of these chemicals actually provide an orientation stimulus for these insect pathogens. The zoospores of Leptolegnia sp. are known to escape from the zoosporangia and swim rapidly before encysting and adhering to the surface of the host or substratum (Alexopoulos 1966,

Petersen 1910, McInnis and Zattau 1982), but no adhering mechanism has been determined for the encysted zoospores.

3. Mode of Infection of *Ae. triseriatus* Larvae by the Zoospores of *Leptolegnia sp.*: and Effect of Starvation on Larval Susceptibility

The mode of infection of *Leptolegnia sp.* has been a subject of generalization, speculation or observation based on post infection fungal masses. Since some pathogenic fungi have been observed to infect their host by producing penetrant or invasive tubes after a period of encystment on the host (Poinar and Thomas 1984, Sweeney 1983, Steinhaus 1949, Burges et al. 1981), it has been generalized that *Leptolegnia sp.* achieves its infection through this mode. Petersen (1910) speculated that the fungus enters its host through the oral opening. McInnis and Zattau (1982) identified two methods by which the zoospores infect their hosts, one by cuticular encystment of secondary zoospores, and two by germination of zoospore cysts in the alimentary canal. They observed that the zoospores infect their hosts by encysting on the cuticle in the regions of the thorax, abdomen and anal papillae.

Following encystment, germ tubes were produced for penetration into the hemocoel where rapid mycelial growth ensued. They further stated that younger larvae were also

observed feeding on encysted zoospores in the media and becoming infected through the midgut, and that midgut infection appeared to be the most common route of invasion of first and second larval instars. Clements (1963) cited a number of ways in which mosquito larvae feed, the most common of which was filter feeding and browsing on fungi and other submerged plankton. Horsfall (1955) described filter and mandibulate feeding as patterns by which Ae. triseriatus larvae ingest suspended and attached materials as they cruise in the water. Such observations were supported by Fish and Carpenter (1982) who observed that this aedeian species feeds on microorganisms in its aquatic environment. Sweeney (1983) noted that the ingestion of conidia and subsequent invasion of germinated conidia in the midgut was the most common mode of infection of Culex quinquefasciatus and Anopheles hilli by a pathogenic fungus Culicinomyces clavisporus.

Induction of mycosis as a pest suppression measure is so much in its infancy that several aspects that might affect such a process have not yet been researched. The suspicion that mosquito larvae become infected by leptolegnial zoospores through ingestion has not been documented through independent research on such observed phenomenon. Consequently, there has been no record on the

impact of starvation or overfeeding on the ability of the larvae to filter feed on the encysted spores or gnaw at the hyphal or sporangial filaments.

4. Effect of Storage Temperature and Storage Period on the Viability and Pathogenicity of *Leptolegnia sp.*

There has been no documentation on the effects of storage on *Leptolegnia sp.* or on the effect of temperature on its pathogenic potentials. However, Johnson (1956) reported that the genus, *Achlya*, (Saprolegniaceae) cultured on hempseed can be preserved in jars filled 2/3 with sterile distilled water and stored at room temperature, or 8°C for 14-28 months with split hempseed replenished every 6 or 8 months. Also that *Achlya* cultured in slants and covered with sterile mineral oil to about 1 cm above the agar can be stored upright at room temperature or at 9°-12°C. Research has been conducted on storage of other pathogenic fungi. Daoust et al. (1983) evaluated the viability and percentage germination of the conidia of *Metarhizium anisopliae* after a period of storage in different types of oils for 2 months, 6 months and 12 months and at temperatures of 4°, 19°, 20° and 26°C respectively. Soares and Pinnock (1984) investigated the

effect of temperature on germination, growth and infectivity of Tolypocladium cylindrosporum infecting mosquito larvae.

5. Pathology and Histopathology of Ae. triseriatus Larvae Infected by Zoospores of Leptolegnia sp.

The disease caused by Leptolegnia sp. on infected mosquito larvae has been characterized as "coelomomycosis" with involvement of other tissues and organs (McInnis and Zattau 1982). They observed that some of the organs involved were the midguts, which became engorged as the larvae grazed heavily on the encysted zoospores. These cysts later germinated in the posterior midguts and occasionally in the colon and rectum, producing germ tubes that penetrated through the gut walls into the hemocoel, where mycelial masses developed. Tissue involvement was depicted by a cuticular layer which became darkened in reaction to the wound made by the invasive tube of the encysted spore that penetrated the cuticle into the hemocoel. Other tissues were suspected of becoming involved as coelomomycosis progressed.

Literature on the internal morphology and anatomy of early instars of mosquito larvae are not available at this time but references by Clements (1963), Wigglesworth (1984) and Snodgrass (1935, 1959) provide valuable illustrations

that might enhance the interpretation of the internal morphology and anatomy of any immature insect.

References on histopathology of leptolegnial infection are lacking at the present time. There have been no light microscopic or ultrastructural studies done on the tissues or organs damaged by leptolegnial infections. However, Steinhaus (1949, 1963), Lipa (1975), Poinar and Thomas (1984) and Weiser (1969) provided very good descriptions and illustrations on the diagnosis of various pathological changes in insects that have been infected by fungi and other pathogens.

Histopathological and ultrastructural studies have been made on larval mosquitoes infected by bacteria, Bacillus sphaericus (Davidson 1979) and Bacillus thuringiensis (Lahkim-Tsror et al. 1983, Percy and Fast 1983), mermithid nematodes, (Bailey and Gorden 1973, Schmidt and Platzer 1980), and other pathogenic fungi. Hepburn (1976) noted a cross section of Metarhizium anisopliea in an ultrastructural preparation of the integument of an elaterid larva. Sweeney (1983) described ultrastructural studies of fourth instar Culex quinquefasciatus and Anopheles hilli infected by a Deuteromycetes fungus, Culicinomyces clavisporus. Adhering mechanisms of the conidia and germ tube, germination of

conidia and invasion of the penetrant tube through larval integument were documented with both scanning and transmission electron micrographs. Procedures for preparing these pathological tissues for electron microscopic studies were also described.

Histological and histopathological techniques on mosquitoes are correspondingly scanty. Where available, the bulk of such literature refers to the adult mosquitoes, pupae or fourth larval instars. Nevertheless, Barbosa (1974) has compiled various histological techniques for light microscopic studies of different parts of insects. Some techniques found in a text by Humason (1972) are applicable to investigations on insect histology. The currently preferred procedure for preparing animal tissues for histological, pathological and histopathological studies with light microscope involve the use of a plastic embedding medium, glycol methacrylate (GMA) instead of paraffin embedding medium (Bennet et al. 1976, Troyer 1980). Tissues embedded in GMA, sectioned with a glass knife mounted on a Sorvall JB-4 microtome, were found suitable for quick staining with any histological and histochemical stains without rehydration and dehydration or removal of the embedding medium, as were necessary with paraffin (Bennet et al. 1976, Troyer 1980).

For scanning electron microscope (SEM) studies, the general procedure of fixing with glutaraldehyde, dehydrating in a series of graded alcohols and drying by the critical point method has been found useful for arthropod studies (Hooper et al. 1979). Grodowitz et al. (1982) found that prefixation in super skipper followed by post fixation in Carl's solution was effective in preparing muscid larvae for scanning electron microscopy. Mosquitoes have been prepared for transmission electron microscopy (TEM) by fixing the specimens in varying concentrations of glutaraldehyde and postfixing in osmium tetroxide, both in phosphate buffer at pH 7.4, rinsing in phosphate buffer pH 7.4, dehydrating in a graded series of ethanol and embedding in Spurr-Mollenhauer resin or Epon 812 to produce blocks that were sectioned with a glass knife mounted on Sorvall MT-2 ultra microtome. Such sections collected on grids could be double stained with uranyl acetate and lead citrate (Harris 1962, Hooper et al. 1979). Sweeney et al. (1983a), working with mosquito larvae, described some variations to the above procedures. References on interpretation of insect ultrastructures, though primarily on the adults, have been provided in texts by Harris (1962), Neville (1970), Hepburn (1976), Akai and Morohoshi (1982), King and Akai (1982, 1984). Asakura (1982)

provided good TEM micrographs of the hindgut epithelium in larvae of a seawater mosquito. Travland (1979a), Sweeney (1979) and Sweeney et al. (1983a) have presented micrographs of some structures in mosquito larvae invaded by fungal pathogens.

MATERIALS AND METHODS

A. Colony of *Aedes triseriatus* (Say)

The eggs of *Aedes triseriatus* Walton strain were supplied from a colony maintained by the Vector Biology Laboratory at the University of Notre Dame, South Bend, Indiana. These eggs were hatched in distilled water, deoxygenated with autolyzed yeast (Difco) in the ratio of 1:1000 ml, aged overnight and well aerated prior to submerging the paper with a few hundred eggs attached. The eggs were brushed off the paper with a fine soft tooth brush, dispersed in the water medium and the paper discarded.

The larvae were reared in white enamel pans 10" x 16" x 3" containing 3000 ml of distilled water. On the first day of emergence, the larvae were fed with a pinch of ground Tetramin^{R} baby fish food. Subsequent feedings until pupation were with a few grains of the same ground Tetramin^{R} baby fish food shaken onto the water. The number of grains was gradually increased as the larvae got older and bigger. The larval pans were covered with sturdy plastic bordered with wood panelling to prevent evaporation and ward off intruding arthropods from the insectory. The larvae were reared at a temperature range of 25-28^{o}C.

Pupae were routinely removed with fine nylon mesh and put into a crystallizing bowl about half filled with water. The bowl was placed in the cage for adults to emerge.

Adults were maintained in 24" x 24" x 24" Gerberg's collapsible cages in an insectory where the temperature ranged from 21-26°C and the relative humidity ranged from 70-100%, with a 16 hour photoperiod which included 1 hour dusk and 1 hour dawn periods of diminished light. The adults were provided with 10% sucrose solution soaked in dental cotton plugs (wicks) which protruded from 50 ml Erlenmyer flasks suspended in the cages with thin metal wires. The sucrose solution was changed weekly. A guinea pig obtained through the Michigan State University Small Animal Care Service served as a blood meal source for the females. A restraining device made from a half gallon size plastic milk carton supported with plastic netting and corks was used to introduce the guinea pig into the adult cages. This device helped to protect the guinea pig's face, and also made the guinea pig remain steady while the female mosquitoes fed on its shaved, exposed back. Blood meals were provided every five days. The mosquito cages were placed on trays supported by rubber stoppers in petri dishes filled with mineral oil to protect the mosquitoes from predaceous ants.

The females were allowed to oviposite in a 600 ml beaker in which 9.0 cm size Whatman filter paper was placed at the bottom while the side was completely lined with 10mm strip cut from size 50.0 cm Reeve angel^{R} grade 201 filter paper. The beaker was half-filled with 300 ml of distilled water. The eggs were collected weekly, stored in bottles and kept in the insectory. One to three weeks were allowed for the eggs to maturate before hatching.

B. The Guinea Pig

The guinea pigs were maintained in cages in a small animal room and fed with guinea pig chow rationed according to the weight of the guinea pig. The cages were provided with water which drips on contact from an inverted stoppered-tube animal bottle. The water was changed twice weekly, and the animal bedding was changed weekly.

C. General Laboratory Practices

All culture and inoculating media used in the course of this research were sterilized. The glassware, autoclavable software and the used or remains of media, fungi and infected mosquito larvae needed to be sterilized before being disposed of. All sterilization was done by autoclaving at 120^{o}C and 15 pounds per square inch for 20 minutes, using American Sterilizer model 57CR. All

sterilized inoculating media were allowed several hours to cool before use. All glassware used for experiments were first washed in Linbro^{R} 7X cleaning solution, rinsed several times in tap water followed by final rinse in distilled water for 3-5 times.

The mosquito larvae used for all experiments were randomly selected from a colony of two-day old larvae, hatched in larval pans already described. The selected larvae were always bathed twice in distilled water before being used for any experiment.

D. Culture of *Leptolegnia sp.*

Leptolegnia sp., an unnamed species of aquatic fungus, was obtained in an agar slant from the American Type Culture Collection, catalogue number 36191. The fungus was transferred onto plates and slants of corn meal agar prepared from 15g of corn meal agar (Difco) in 1 liter of water, steamed for 10-15 minutes to melt the agar, and autoclaved for 20 minutes. The cultures were maintained at 25^{o}C and frequently transferred to fresh culture media. The agar plates were sealed with Time Labels^{R} tape to prevent dehydration and contamination.

The stock culture was established by cutting 0.5cm agar blocks from the edge of an actively growing colony. The hemp seeds, *Cannabis sativa*, boiled, split into two

halves with a razor blade, and autoclaved , were used as bait for the fungal culture. These sterilized half split hemp seeds were affixed to the edge of the block containing young fungal hyphae. The half split hemp seeds with fungal blocks were transferred to petri dishes containing 10 ml of sterilized double distilled water. After 3 days, the fungi became established on the hemp seeds, which were then removed from the petri dishes, passed through a single wash in sterilized double distilled water, and transferred to a Wheaton jar containing 20-30 ml of sterilized double distilled water. The stock culture was incubated at temperature of 25^{o}C for 1 day and then stored in the refrigerator at a temperature of 5^{o}C. Fresh sterilized double distilled water and a sterilized half split hemp seed were used to replenish the stock culture every 3 months. Sterile mycological techniques were employed in making all transfers.

E. Preparation of Fungal Inoculum

A few hyphae were removed from the stock culture and washed in sterilized double distilled water. After blotting off excess water, the hyphae were streaked on corn meal agar plates prepared as described in section D. The plates were sealed and incubated for 3 days. On the third day, 0.6 cm disks were cut from the margins of the new

growing hyphae. Sterilized half split hemp seeds were placed next to the edge of the agar disks with fungal hyphae. These agar disks with half split hemp seeds were transferred into sterile 10 ml double distilled water in standard sterile petri dishes. Eight half split hemp seeded-disks were put in each petri dish, and incubated at 25^{o}C for 3 days until fungal colonies became established on the split hemp seeds. On the third day of incubation, the dishes were examined and the split hemp seeds with fungal colonies established on them were removed from the disks and washed in sterilized double distilled water. They were then transferred to 10 ml of fresh, sterilized, double distilled water in standard petri dishes and incubated at 25^{o}C for 3 additional days to allow for the formation of sporangia and zoospores.

On the third day of incubation the petri dishes with fungal colonies on split hemp seeds were examined under the compound microscope. Colonies with sporangia at the end of the hyphae were transferred to fresh sterilized 10 ml double distilled water in standard sterile petri dishes for washing. After two washes in sterilized double distilled water, the fungal colonies on split hemp seeds were ready for use as inoculum. Each fungal colony on half split hemp seed containing sporangia and matured hyphae was regarded as an inoculation unit (inoculum). An illustration of four such units is shown in Figure 1.

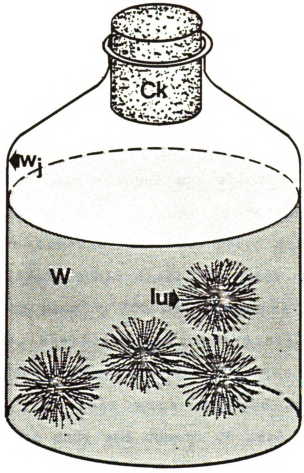


Figure 1. Schematic diagram of four inoculation units placed in a Wheaton jar containing sterilized double distilled water. (Iu-Inoculation unit, W=water, Wj=Wheaton jar, Ck=Cork)

EXPERIMENTAL PROCEDURE

A. Bioassay of *Aedes triseriatus* Infected by *Leptolegnia sp.*

Five 133 x 102 mm polycarbonate Nalgene^{R} cylindrical jars with matching lids were filled with 600 ml of double distilled water, autoclaved and allowed to cool. Four inoculation units that had well developed hyphae with sporangia were removed from the petri dishes and washed once in sterilized double distilled water. Infection was accomplished by placing 100 bathed larvae in each jar of 600 ml cooled, sterilized, double distilled water and introducing four washed inoculation units into each such larval jar. The jars were covered and set aside. Observations were made and counts of dead mosquito larvae were taken at 12, 24, 48 and 72 hours after inoculation. The dead larvae were placed in concavity slides and examined under the compound microscope for signs of infection by the fungus, *Leptolegnia sp.*

A total of 500 larvae were inoculated in each replication. The experiment was repeated four times. The controls for this experiment were 100 larvae placed in similar containers with the same volume of sterilized

double distilled water. Four sterilized, half split hemp seeds that were not exposed to fungal infestation were washed and placed in these jars. Observations and counts were conducted at the same time, in a similar manner. The control also had four replications with 500 larvae per replication.

Data obtained from these experiments were analyzed using Analysis of Variance to obtain an LT_{50}. When variances were not homogenous, transformation was made using LogX.

In the course of conducting the bioassay tests, concurrent observations were made to ascertain the stages in the life cycle of Leptolegnia sp. A matured inoculation unit that had been washed twice in sterilized double distilled water was placed in concavity slide containing unused drops of this sterilized water and examined with American Optical light microscope for the release of zoospores. The actual process of zoospore release and subsequent activities of the released zoospores were observed and documented. The same procedure was repeated with leptolegnial colonies growing out of infected dead mosquito larvae 48 and 72 hours after inoculation. Photographs of the hyphae, sporangia and zoospores as they were being released, were taken on Kodak Panatomic X black and white film with Canon AE-1 single lens reflex 35mm

camera attached to the microscope. Sketches were made of those zoospore configurations that were not photographed.

Further attempts were made to observe more stages of the life cycle or obtain morphological details of some stages through the use of scanning electron microscope (SEM). A sample of water, in which two-day old mosquito larvae and the fungal inoculum had been incubated for 48 hours, was strained and fixed 1:1 (v/v) with 4% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes on ice. It was then passed through a nuclearpore filter, carefully washed in several drops of buffer. With the suspension side up, the filter was floated on buffered 4% glutaraldehyde for additional 30 minutes on ice. It was again washed with many drops of water and blotted slightly dry before being dehydrated in graded series of ethanol at 10 minutes per grade. It was then critical point dried, mounted with adhesive tape, coated and examined as described in #a of the SEM procedure for the pathology and histopathology section of this thesis.

B. Persistence of *Leptolegnia sp.* in Infecting Larvae of *Aedes triseriatus* Under Laboratory Conditions

Five Nalgene jars already described in the previous section were filled with double distilled water that had been autoclaved and cooled. One hundred bathed larvae of similar size and vigor were placed in each of these jars.

Four inoculation units with fully developed sporangia were washed once and placed into each infection jar containing 100 larvae. The jars were covered and set aside. Observations were made and counts of dead larvae were taken 24, 48 and 72 hours after exposure of the larvae to the pathogen. Microscopic examinations were made of dead larvae to ascertain that death was due to the pathogenic fungus, Leptolegnia sp. A total of four replicates, with 500 larvae per replication were made. At 72 hours, after final data had been collected, all larvae and larval remains were removed from the infection jars, autoclaved and discarded. The inoculation units were subsequently removed from the infection jars, washed twice in sterilized double distilled water and placed in standard sterilized petri dishes. The five infection jars were washed clean, rinsed with distilled water, refilled with 600 ml of double distilled water, autoclaved and cooled for the next use.

Six days after the completion of the first 72 hour exposure period, another batch of 100 healthy and vigorous two-day old larvae were bathed and introduced into each jar of cooled sterilized water. The fungal inoculation units that had been removed from each of the infection jars in the previous experiment, were placed in each jar. The jars were set aside and examinations and counts of dead larvae again were made 24, 48, 72 hours post inoculation. This

procedure was repeated three additional times using the same inoculation units. Thus, mosquito larvae were exposed to each inoculation unit over an elapsed period of 27 days to demonstrate the persistent pathogenicity of the fungus against five different batches of Ae. triseriatus larvae. The experiment had four replications. As a control for this experiment a similar set up was used but employed the use of freshly prepared inoculation units.

Data obtained from this experiment was analyzed using SAS Analysis of Variance; General Linear Models Procedure. Both Duncan's multiple range test and Scheffe's test was run on percent means to determine the significant differences at 0.05 level. When variances were not homogenous, transformations were made using Arcsin.

C. Orientation of Zoospores of Leptolegnia sp. Towards the Larvae of Aedes triseriatus

Observations from previous experiments had indicated that vigorous and actively moving zoospores were more readily produced by the sporangia on fungal colonies growing on dead mosquito larvae than from sporangia developed from the split hemp seeds. In order to obtain such actively moving zoospores for this orientation experiment, the mosquito larvae were inoculated in advance. Adequate time was allowed for sporangia, bearing numerous zoospores, to develop in these larvae. These sporangia

were removed for the orientation experiment. For each replication, 25 two-day old bathed larvae were placed in a 150 ml crystallizing bowl containing 100 ml of sterilized, cooled, double distilled water. The larvae were exposed to one washed freshly prepared fungal inoculation unit. The bowls were covered and incubated for 72 hours to allow development and maturation of the sporangia.

The Pyrex^{R} tube assay technique, a modification of the capillary tube assay of Van Houten (1977), was employed to provide the zoospores with a choice of surfaces on which to orientate, encyst and adhere. Three sets of six mm Pyrex^{R} glass tubes were cut into lengths of 20 mm, washed and autoclaved in double distilled water for 20 minutes. One set of tubes was filled with a larval homogenate prepared by blending 250 second instar larvae of Ae. triseriatus in a glass homogenizer. Twenty-five ml of larval homogenate were mixed with 25 ml of liquid plain agar to create the consistency of a normal agar growth medium. The plain agar was made from 20 gm Difco agar in 1 liter of distilled water, then steamed for 10-15 minutes to melt the agar. The second set of tubes was filled with plain agar medium. Both the homogenate and the plain agar media were autoclaved for 20 minutes before filling the tubes. To create the desired volume, one end of each tube was temporarily sealed with sterilized strips of aluminum foil.

The homogenate and plain agar were pipetted into the tubes under sterile conditions and allowed to solidify at 25°C before the aluminum strips were removed to expose both ends of the filled tubes. When the media in the tubes congealed, a surface area inside each tube was exposed so the zoospores could contact either the surface of the media or the glass tubing for encystment and adherence. The third set of tubes was left empty so that the zoospores could swim in, out or adhere and encyst on the tube surface.

The three sets of tubes were randomly placed in one end of the 250 ml crystallizing bowls containing 200 ml of sterilized double distilled water. With the aid of a light microscope, the infected larvae, killed by the fungal hyphae which projected from all parts of the body, were examined for matured sporangia ready to release zoospores. Five sporangia with motile zoospores were carefully detached from the bulk of the cadaver with a flamed dissecting needle, washed, and transferred to the free end of each crystallizing bowl opposite the tubes. The experiment had a randomized complete block design with three treatments, sixty observations and four replications. The tubes with the homogenate media were the experimental groups, and controls included the tubes with plain agar and the empty tubes. After 48-72 hours, during which the released

zoospores could swim, encyst and adhere to a preferred surface, the number of encysted zoospores on the tubes were counted under the light microscope. Counts were made on three surface areas of each tube: the two ends from which the media were exposed, and through which the zoospores could enter the tubes, and the central portion of the tubes including the sides on which the zoospores could adhere or enter and encyst directly on the media. A replica of the experimental set up, using petri dishes, to illustrate random placement of the tubes and the resulting encystment is shown in a schematic diagram in Figure 2.

Data obtained from this experiment were analyzed using analysis of variance. When variances were not homogenous, transformation was made using Logx.

D. Mode of Infection of *Ae. triseriatus* Larvae by the Zoospore of *Leptolegnia sp.*

These experiments were set up to investigate the mode of infection by *Leptolegnia sp.* on *Ae. triseriatus*.

One hundred two-day old bathed larvae were placed in a 300 ml crystallizing bowl containing 250 ml of sterilized, cooled, double distilled water. These larvae were treated with two washed, freshly prepared inoculation units. The infection bowl was covered and incubated at 25°C. Six hours after inoculation, 10 larvae were taken out and washed twice in sterilized double distilled water in a

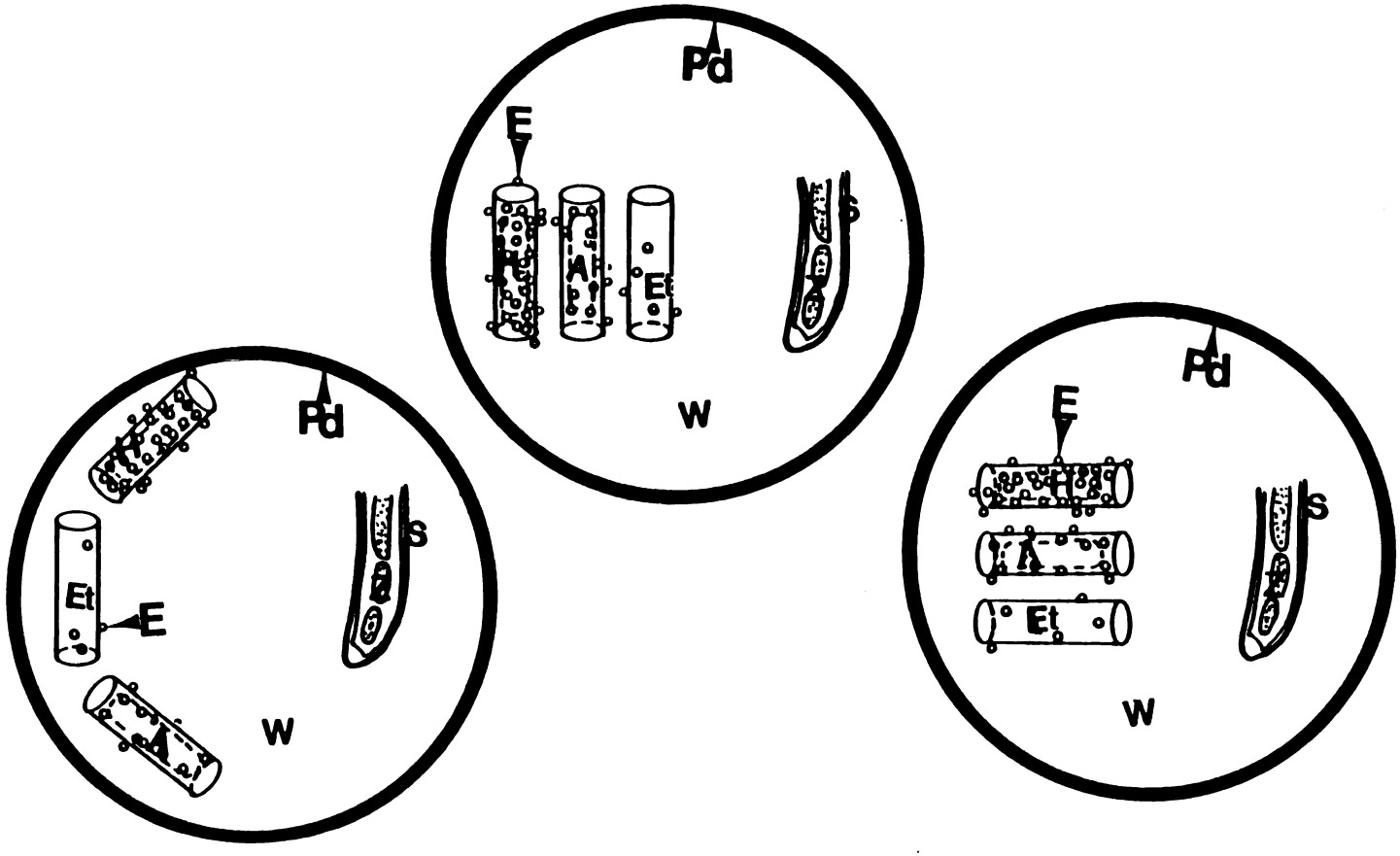


Figure 2. Schematic diagram of zoospore encystment on or adherence to glass tubing. (A=Agar, Et=Empty, H=Homogenate, S=Sporangia, Z=Zoospore, W=Water, Pd=Petri dish, E=Encysted zoospores, ⬭=Glass tubing)

standard sterile petri dish. They were fixed in 10% buffered formalin for 30 minutes, washed in phosphate buffer pH 7.3 for 10 minutes, and dehydrated in a graded series of ethyl alcohol for ten minutes each.

These larvae were then placed two at a time in concavity slide containing ethanol. Following examination under a light microscope for symptoms of leptolegnial infection, the larvae were dissected under the Bausch and Lomb dissecting microscope for observation of the hemocoel, gut, trachea, and other visceral organs. After examination and dissection the specimens were cleared with a 50:50 solution of methyl salicylate and xylene for 3 minutes, followed by xylene for 1 minute and mounted in Kleermount^{R}. The above procedure was followed with 10 dead and 10 moribund larvae taken out after exposure times of 11, 15, 24 and 48 hours. Seventy-two hours after exposure to the pathogen the remaining 10 larvae were removed from the infection jar, washed, fixed, dehydrated, stained, cleared and mounted without dissection. All specimens were stained with Lactophenol Cotton Blue to highlight hyphal structures, and with crystal violet to highlight spore structures. This experiment was repeated four times. Photographs were taken to illustrate the development of leptolegnial infection within the body of Ae. triseriatus larvae and also depict the target organs for infection.

Based on data obtained from the preceding experiments and observations, efforts were made to document the hypothesis that actual infection starts from inside the body of the larvae, initiated by leptolegnial structures that have gained entrance either through ingestion or direct entrance via any possible opening.

One freshly prepared inoculation unit was washed in sterilized double distilled water and stained with 1% crystal violet for 5 minutes. Thirty two-day old larvae of Ae. triseriatus were bathed twice in distilled water and placed in a 150 ml crystallizing bowl containing 100 ml of sterilized, cooled, double distilled water. These larvae were exposed to the stained inoculation unit, covered and incubated as per previous experiments. Six hours after treatment the infection bowl was examined for larvae that had some crystal violet pigmentation within them. Ten such pigmented larvae were taken out of the infection bowl for light microscope observation and subsequent dissection under the dissecting microscope. Twelve hours after exposure, another ten pigmented larvae were taken and the procedure of observation and dissection already described was followed. Fifteen hours after exposure the remaining ten larvae were recovered from the infection bowl and treated as their predecessors. This experiment was repeated four times. Photographs were taken to portray the

extent of pigmented leptolegnial remains ingested by the larvae within a given time.

E. Effect of Starvation on the Susceptibility of *Ae. triseriatus* Larvae to Infection by *Leptolegnia sp.*

Observations from previous experiments indicated that the larvae actively feed on the fungi. To determine whether such feeding action was induced by hunger or a natural behavior that can happen under any circumstance, the following experiment was undertaken.

Five 100 x 50 mm crystallizing bowls with matching covers were filled with 250 ml of double distilled water, autoclaved and cooled. A population of two-day old larvae was maintained without any food since hatching. Fifty larvae of similar size and vigor were selected from this population of starved larvae, bathed and transferred to the water in each of these infection bowls, and exposed to two inoculation units. The bowls were covered and set aside on a laboratory counter at a temperature of 25°C. Observations were made and counts of dead larvae taken 24, 48 and 72 hours after inoculation. The dead larvae were examined for infections with *Leptolegnia sp.* This experiment had four replications with five samples per replicate and each sample had 50 larvae, making a total of 250 larvae tested per replicate. Controls employed an equal number of fed larvae.

In the first part of this experiment, the inoculation unit was left in the infection bowl throughout the 72 hours of the experiment. Based on the results obtained, a second experiment was set up to determine if a continuous availability of the inoculation unit had any effect on the susceptibility of the starved larvae to infection by the zoospores. The second experiment used 50 bathed, starved larvae and two inoculation units in 250 ml of autoclaved, cooled, double distilled water in 100 X 50 mm crystallizing bowl that was covered and incubated at room temperature. After 24 hours, the two inoculation units were removed from the infection bowls. Observations and counts of larval mortality with leptolegnial symptoms were made 24, 48 and 72 hours after initial exposure. This experiment had four replications with five samples per replicate, and each sample had fifty larvae, bringing the total number of larvae tested per replication to 250. Again, fed larvae were used as a control in a similar experimental set up.

Data for both parts of the experiment were analyzed using SAS Analysis of Variance, and Duncan's multiple range test. Where variances were not homogenous, transformations were made using LogX, and Arcsin.

F. Effects of Storage Temperature and Storage Period on the Viability and Pathogenicity of *Leptolegnia sp.* Infecting the Larvae of *Ae. triseriatus*

Three-hundred Wheaton jars with tight-fitting corks were filled with 27 ml of double distilled water, to which 0.05gm of Chloromycetin per 1000 liter of distilled water was added to inhibit bacterial growth, then autoclaved and allowed to cool. Two inoculation units, washed in sterilized double distilled water, were transferred to each jar. The jars were flamed and tightly corked. The jars were placed in plastic bags that were snugly tightened. One hundred of these bagged jars were stored in the refrigerator at a temperature of 5°C. Another one hundred were placed in small paper cartons and stored on the laboratory shelf at a temperature of 25°C. Fifty of these bagged jars were placed in the refrigerator freezer at a temperature of 0°C. The remaining 50 jars were placed in a deep freezer at a temperature of -20°C.

Another 100 Wheaton jars with fitting corks were filled with 27 ml of a sterilized mixture of water and 10% glycerol to prevent protoplasmic collapse at lower temperatures. Two washed inoculation units were also placed in each of these jars and allowed to grow for 24 additional hours at room temperature. Fifty of these glycerol-conditioned infection jars were also bagged in plastic and placed in the refrigerator freezer at a temperature of 0°C

while the remaining 50 were bagged and placed in a deep freeze at a temperature of -20°C. The jars to be stored in zero and subzero temperatures were passed through chambers with decreasing temperature to the ultimate one in which they were stored. Each storage unit was equipped with a thermometer whereby the temperatures were monitored regularly. Records were kept on temperature and months in storage. The designated periods of storage were 3, 6, 9 and 12 months for each temperature.

At the third month of storage, 25 Wheaton jars, each of which contained 2 inoculation units, were randomly taken out for infection experiment from the storage units that had 100 jars and 12 jars were taken out of the units that had 50 jars. Five 100 X 50 mm Pyrex^{R} crystallizing bowls were filled with 250 ml of double distilled water in each bowl, autoclaved for 20 minutes and allowed to cool for several hours. Larvae of similar size and vigor were selected randomly from a colony of two-day old larvae of Ae. triseriatus and bathed twice in distilled water. Fifty of these bathed larvae were placed in the cooled sterilized water in each bowl. Five out of the 25 and 2 out of the 12 jars containing inoculation units stored at different temperatures were opened under aseptic conditions, and the inoculation units transferred to freshly sterilized double distilled water in marked standard sterile petri dishes.

These units were microscopically examined for signs of viable protoplasm and the presence of developed sporangia. Good, viable inoculation units were selected, washed twice in double distilled water and placed in the crystallizing bowls at the rate of two units per bowl of fifty larvae. The bowls were covered and incubated at 25°C. Observations were made and counts of dead larvae taken 24, 48 and 72 hours after exposure to the stored pathogen. Microscopic examinations were made of dead larvae to ascertain that death was caused by leptolegnial infection. The experiment had four replicates with five samples containing fifty larvae per sample, per replication for each temperature that viable inoculation units were found. This experimental design made it possible to test a total of 250 larvae per replication per storage temperature for each storage period under study. Agar cultures were also made from a piece of each stored inoculation unit used, to confirm its viability through the period of storage.

The controls for this experiment followed a similar procedure except the inoculation units used were freshly prepared at laboratory room temperature of 25°C and were not subjected to any storage.

At the sixth, ninth and twelfth months, respectively, the same experimental and control procedures were repeated for only units stored at 5°C and 25°C, since they still

sustained viable inoculation units. The same number of replications were used in each experiment.

A randomized complete block design with subsampling was employed in conducting this experiment (Figure 3). Data obtained from the experiment were analyzed using 3 X 2 X 4 factorial SAS Analysis of Variance, multiple comparisons, and Duncan's multiple range test was used to compare the means. Data was analyzed to show the interaction of any two of the three variables (temperature, months in storage, assay time) and all three variables as illustrated in Figure 3.

G. Effect of Storage Temperature and Growth Medium on Viability and Pathogenicity of Leptolegnia sp.

Leptolegnia sp. growing on corn meal agar was transferred onto 20 agar slants of Peptone-Yeast Extract-Glucose (PYG), Corn Meal(CM), and Corn Meal Dextrose Peptone (CMDP), and ten agar slants of each of the above media to which 10% glycerol was added. The slant cultures were allowed three days to become established. On the third day ten slant cultures from each group were filled with sterilized, heavy white mineral oil, viscosity 340-360, and incubated for one more day at laboratory room temperature of 25^{o}C. The slant cultures were divided equally into two groups such that each treatment was represented by five culture slants. One group was stored in the refrigerator

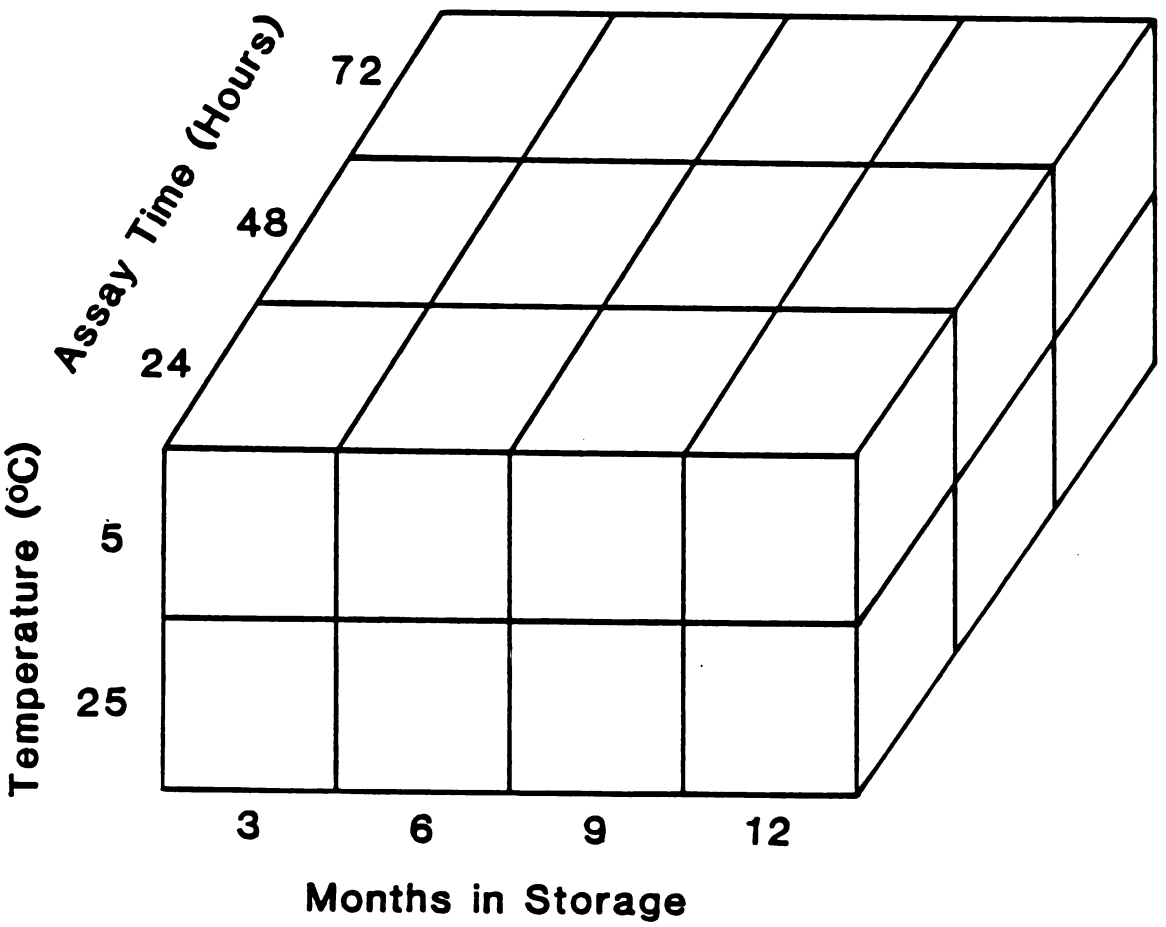


Figure 3. Schematic presentation of the experimental design on effect of storage period and temperature on pathogenicity of Leptolegnia sp. Infecting Ae. triseriatus larvae (3x2x4 factorial).

at a temperature of 5°C, and the other group was stored on the laboratory shelf at a temperature of 25°. Each group was stored for 2 years at designated temperatures.

At the end of the two years, each slant culture was transferred onto a freshly prepared agar slant and allowed to grow, if still viable, or demonstrate a lack of viability. The viable cultures were transferred to CM agar plates and a unit of inoculation prepared from that plate culture. The inoculation units thus obtained were applied to a population of Ae. triseriatus larvae, and larval mortality determined after 72 hours of exposure. Only trial runs were made to determine if pathogenicity would occur. Dead larvae were examined for symptoms of leptolegnial infection. Infectivity tests were not duplicated.

H. Pathology and Histopathology of Ae. triseriatus Larvae Infected by Zoospores of Leptolegnia sp.

Experiments using light, scanning and transmission electron microscopes were conducted to determine the pathological damage that leptolegnial infection inflicts on the organs and tissues of the larvae of Ae. triseriatus.

1. Light Microscopy

Two day old larvae of Ae. triseriatus were exposed to infection by Leptolegnia sp. while others were left

unexposed and used as controls. Twenty-four hours after exposure, some larvae that were dead, moribund and controls were put in three separate vials and fixed in 4% glutaraldehyde in 0.1M phosphate buffer on ice for 2 hours. The fixed specimens were then washed twice in 0.1M phosphate buffer for 45 minutes. All fixed and washed specimens were dehydrated in graded series, 70% 95% and 100% ethyl alcohol for 30 minutes. They were infiltrated with glycol methacrylate (GMA) solution A plus catalyst for two hours, then transferred to a fresh solution A plus catalyst for another two hours.

The specimens were then embedded in an ice mixture of 1 part solution B to 25 parts solution A, plus a catalyst and allowed to polymerize at room temperature. The polymerized GMA blocks were removed from the plastic embedding molds and microtomed with a glass knife mounted on Sorvall JB-4. The serial sections were picked up with fine point forceps as they came off the knife and immediately floated on warm distilled water in a water bath to flatten out. After a few seconds the flattened sections were carefully put on clean microscope slides and the slides were placed on a warm hot plate maintained at a temperature of 50-55^{o}C to dry with the specimens secured to the slides.

Three and five micrometer serial sections were cut from the three specimen samples. The three micrometer sections were stained with toluidine blue to depict fungal structure within the thin section. The five micrometer sections were stained with Periodic Acid Schiff (PAS), not only to depict the presence of fungi in thick sections but also to highlight the various tissues in the insect. Stained slides were allowed some days to dry at room temperature before being mounted in Permount^{R}. Prepared slides were examined with an American Optical light microscope. Photographs were taken on Kodak Panatomic X black and white film with Canon AE-1 single lens reflex 35 mm camera attached to the microscope.

2. Scanning Electron Microscopy (SEM)

Several techniques of SEM were attempted to minimize rupturing of integument and loss of hair and brushes in the two-day old larvae used in this experiment. Only techniques whose micrographs are embodied in this thesis will be described here.

a. Samples of two-day old mosquito larvae that had been exposed to infection by the fungal pathogen for 24, 48 and 72 hours were collected along with the comparable unexposed larvae. These samples were fixed in 4% glutaraldehyde in 0.1M phosphate buffer on ice for 2 hours. Fixed samples were washed for 30 minutes on

ice with two changes of 0.1M phosphate buffer pH 7.2. Dehydration was accomplished with graded ethyl alcohol series of 25%, 50%, 75%, 95% and two changes of 100% at 15 minutes intervals. The samples were immediately critical point dried using a Sorvall critical point dryer with CO_2 as the carrier gas.

Dried samples were mounted on a semi-dried layer of Television Tube Koat^R spread on aluminum stubs. The dried samples were coated with 20-40 mm layer of gold using a Sputter Coater operating at 10 milliamps for 3 minutes. Samples were then examined in an ISI Super III scanning electron microscope. Photographs were taken with Polaroid type 107 positive film.

b. Samples of two-day old larvae used as a control and those exposed to the pathogen for 72 hours were fixed in super skipper solution (Grodowitz et al. 1982) for 15 seconds. Fixed samples were rinsed twice in Carl's solution and then left in Carl's solution for 12 hours. Samples were then dehydrated with graded alcohol series of 30%, 50%, 70%, 80%, 85%, 90%, 95% and 3 periods in 100% for 15 minutes in each concentration. These were critical point dried, mounted on stubs, coated , examined and photographed as described in #a above.

c. Samples of two-day old larvae exposed to the pathogen for 24 hours were dehydrated in graded series of alcohol as described in #a above. Samples were transferred directly from 100% alcohol to liquid nitrogen and allowed to remain there until the bubbles had stopped. This frozen sample was touched slightly with a tip of razor blade chilled in liquid nitrogen. Samples were returned to fresh 100% ethyl alcohol, critical point dried, mounted, coated examined and photographed as described in #a above.

3. Transmission Electron Microscopy (TEM)

Samples of two-day old larvae that were unexposed and those that were exposed to the pathogen for 12, 24 and 48 hours were fixed in 4% glutaraldehyde in 0.1M phosphate buffer on ice for 2 hours. These samples were then washed on ice in 0.1M phosphate buffer pH 7.2 in two changes for 30 minutes each. Post fixation was accomplished in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.2 for 1.5 hours, then a 20 minute wash in two changes of 0.1M phosphate buffer. Dehydration was accomplished with an ethanol series of 25%, 50%, 75%, 95% and two changes of 100% at 20 minute intervals. Samples were kept in 100% ethanol overnight in a refrigerator.

Gradual infiltration was accomplished by use of transition solvents in following ratios of 2 : 1 alcohol :

acetone, 1 : 2 alcohol:acetone, 100% acetone, for 15 minutes in each mixture, followed by 3 : 1 acetone : Spurr's/Mollenhauer resin, 1 : 1 acetone : resin for 1 hour in each mixture on a rotary shaker, and 100% resin overnight on a rotary shaker. Samples were then embedded in Spurr's/Mollenhauer resin mixture (Hooper et al. 1979) in a rubber embedding mold and placed in the desiccator for 24 hours. Samples were then moved within the resin, gas pockets removed from the resin and resin samples placed in an oven at a temperature of 65^{o}C for the samples to polymerize within 48 hours.

Polymerized samples were removed from the molds, allowed to cool and individual blocks mounted on microtome chucks for trimming with sharp razor blade under a dissecting microscope. Ultrathin sections were cut with a glass knife mounted on a Porter Blum MT-2 ultramicrotome. The sections were allowed to float on troughs prepared by attaching Scotch^{R} masking tape to the glass knife. Ribbon sections were collected on 300 mesh, uncoated, copper grids and stained in uranyl acetate, rinsed and double stained in Reynolds' lead citrate (Reynolds 1963). Sections were examined on a Philips 201 transmission electron microscope operated at 60 KV. Micrographs were taken on 3.25" x 4" Kodak image plates #4489, developed and printed on Kodak Polycontrast R/C II paper.

RESULTS

A. Bioassay of *Leptolegnia sp.* on *Ae. triseriatus*

The bioassay test was conducted to determine the effectiveness of the inoculation unit (hyphae with sporangia growing on half split hempseed) to release zoospores that would infect *Ae. triseriatus* larvae and also to determine the rate and potential of zoospores to induce mortality in the larval population.

The bioassay experiment determined the ability of split hempseed inoculation units to release zoospores, over time, that were lethal to two-day old larvae of *Ae. triseriatus* within certain periods of time. This inoculation technique was effective in producing zoospores that killed all the larvae tested within 48 hours. Fifty percent of the larvae tested were killed within an average of 11.5 hours. Increased pathogenicity, as measured by the mortality produced, occurred with an increase in exposure time until 99.95% mortality was attained within 48 hours (Table 1). The highest rate of pathogenicity occurred within 24 hours when the total mortality was 96%. The remaining 4% were killed between the 24^{th} and 48^{th} hour. The variations at 12 hours were due to variations in time

of development of sporangia and zoospores. Data obtained from this bioassay experiment was used for plotting a mortality curve to be used for evaluating the effectiveness of Leptolegnia sp. to parasitize the larvae of Ae. triseriatus in subsequent experiments (Figure 4).

Table 1. Mortality of two day old larvae of Ae. triseriatus assayed for pathogenicity by Leptolegnia sp.

Number of Replications	%Larval Mortality per Assay Time (Hours)			
	12	24	48	LT_{50}
1	72.4	95.3	100.0	4.2
2	29.2	96.0	100.0	15.0
3	35.2	97.6	99.8	13.8
4	44.2	96.6	100.0	12.9
X	45.25	96.38	99.95	11.5

The comparative observations of the matured inoculation units and the leptolegnial colonies emerging from infected larval cadaver showed similar stages in the life cycle of this fungal pathogen. Any of the long, delicate, non-septate hyphae measuring 7 micrometers in diameter can develop into a zoosporangium, measuring 11-15 micrometers long, and 9 micrometers wide, containing a single row of zoospores and having a pore at the tip. It

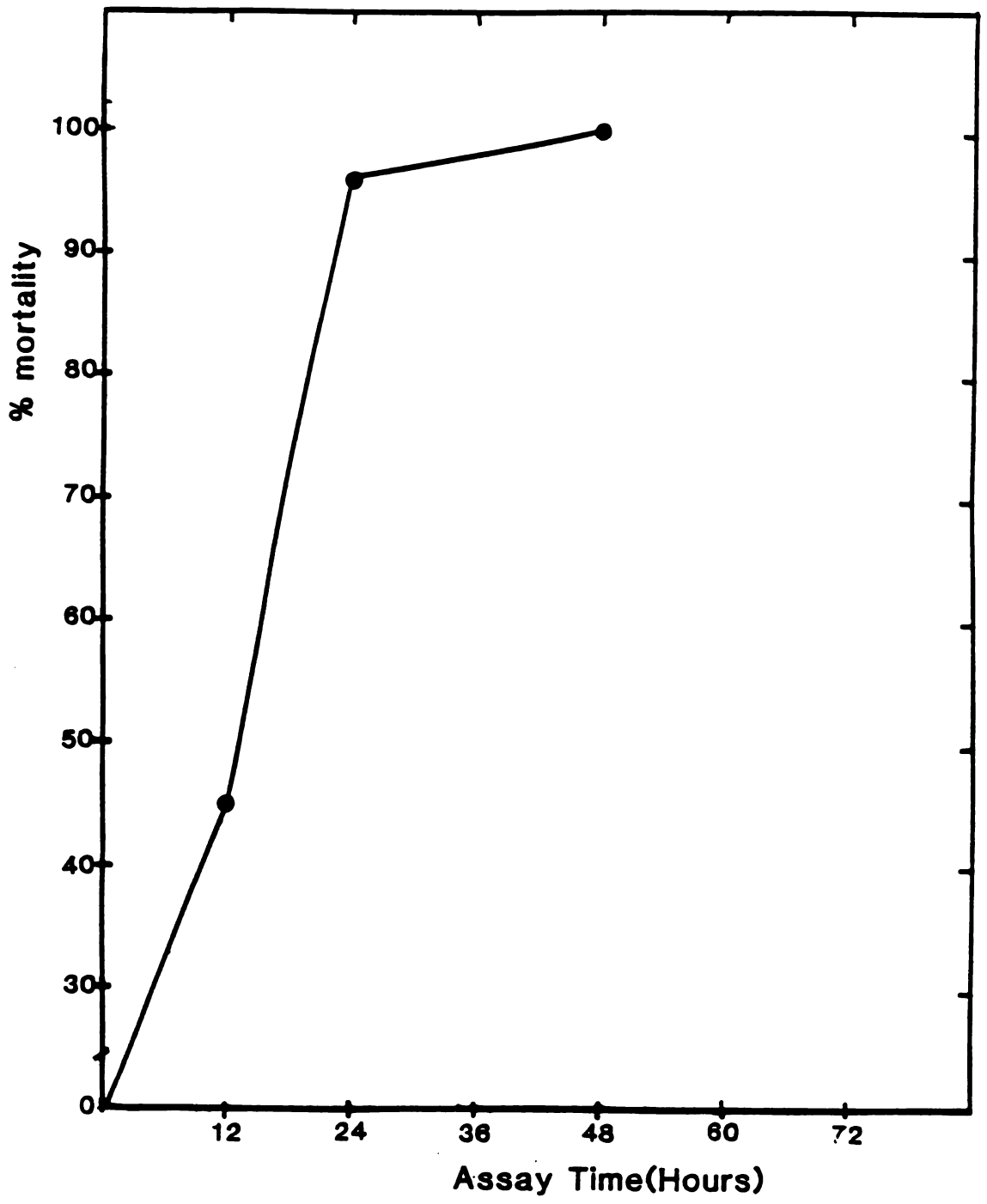


Figure 4. Mortality rate of two-day old larvae of Ae. triseriatus infected with zoospores of Leptolegnia sp.

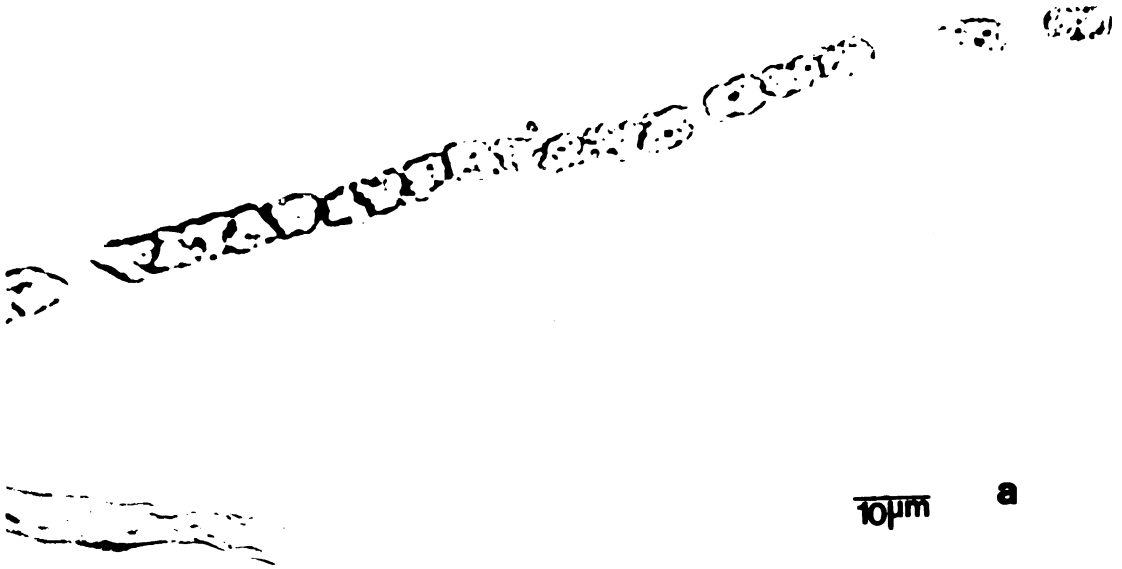
was observed that the zoospores emerged from the zoosporangia (Figure 5A) in straight string through the pore, each having a rod-shaped configuration and measuring 13 micrometers long and 2.5 micrometers in diameter. Within a very short time each zoospore curved and eventually formed a V-shape. After a few other brief configurations, the pear-shape was formed. Under this laboratory observations the pear-shaped zoospore swam for about 5 minutes before encysting into a thick-walled, ornamented primary spore of 20-24 micrometers. After a few minutes of encystment, the primary spore indented itself to form a curve at the anterior tip, and then changed into a couple more configurations before forming a semi grape-seedlike configuration which swam for a longer time before encysting to form a smaller sized spore measuring 16-20 micrometers. An illustration of the stages in the asexual life cycle of this species, as observed in this study, is represented in Figure 5B.

Although the sexual life cycle for this unnamed species has not been worked out, an examination of the suspension from the nuclearpore filter, under SEM, indicated the presence of sexual reproductive structures similar to literature citations for named species of Leptolegnia. The SEM preparation of this leptolegnial

Figure 5A. Some asexual reproductive structures in the life cycle of Leptolegnia sp.

a. Zoosporangium (sporangium)
Note the chain of zoospores within the sporangium.
b. Newly released zoospores from the sporangium

Note the rod-shaped configuration.


10μm
a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b

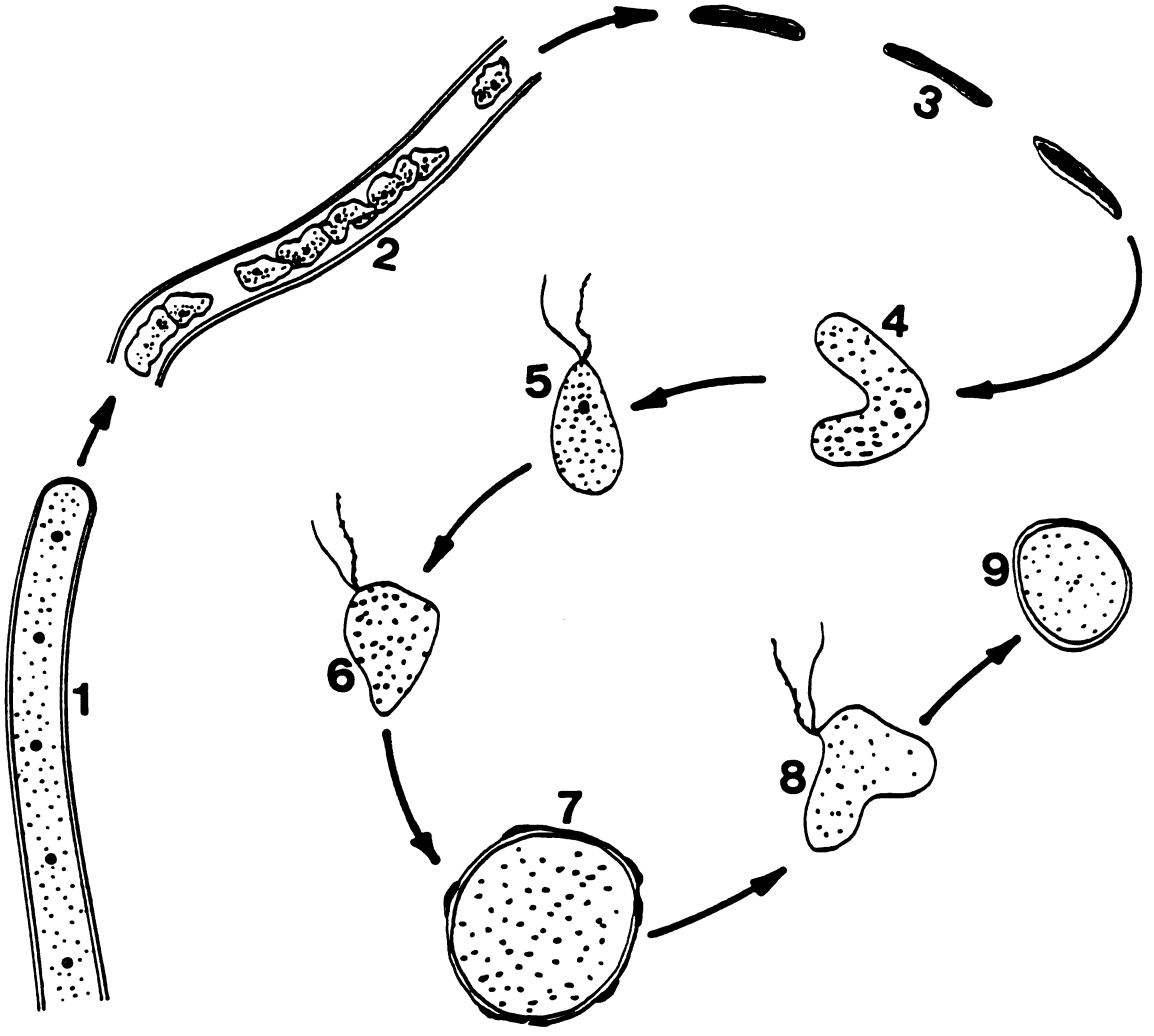


Figure 5B. Diagrammatic presentation of the life cycle of Leptolegnia sp.

1. Hypha
2. Sporangium
3. Newly released zoospore (in rod configuration)
4. Second stage in the alteration of primary zoospore (in V-shaped configuration)
5. Third stage in the alteration of primary zoospore (oval-shaped configuration)
6. Fourth stage in alteration of primary zoospore (pear-shaped configuration)
7. Encysted primary zoospore
8. A stage in alteration of secondary zoospore (grape-shaped configuration)
9. Encysted secondary zoospore

suspension showed a distinct female gametangia that developed into oogonia borne on lateral branches, and ranging in size from 30-37 micrometers. One oogonium seemed to be enclosed, while the other was fully opened. The opened oogonium appeared to have an oospore containing many oil droplets. The enclosed oogonium showed a broken segment, probably damaged during SEM preparation. An antheridium appeared to be laterally attached to each oogonium. SEM micrograph obtained from this observation is presented in Figure 5C.

B. Persistence of Leptolegnia sp. in Infecting the Larvae of Ae. triseriatus

This experiment tested the ability of the hempseed inoculation unit to produce zoospores that persisted and remained pathogenic through five successive batches of mosquito larvae. The hempseed inoculation unit remained viable and continued to produce zoospores that killed Ae. triseriatus larvae for 27 days. All the larvae exposed were killed within 72 hours (Table 2). These inoculation units induced an average mortality rate of 78% within 24 hours, 97% within 48 hours and 98% within 72 hours after exposure to the larvae, for all batches tested (Figure 6).

Figure 5C. Some sexual reproductive structures in the life cycle of Leptolegnia sp.

a. An opened oogonium with appressed antheridium
   Note the large spherical oospore-like structure and the oil droplets
b. An enclosed oogonium with appressed antheridium

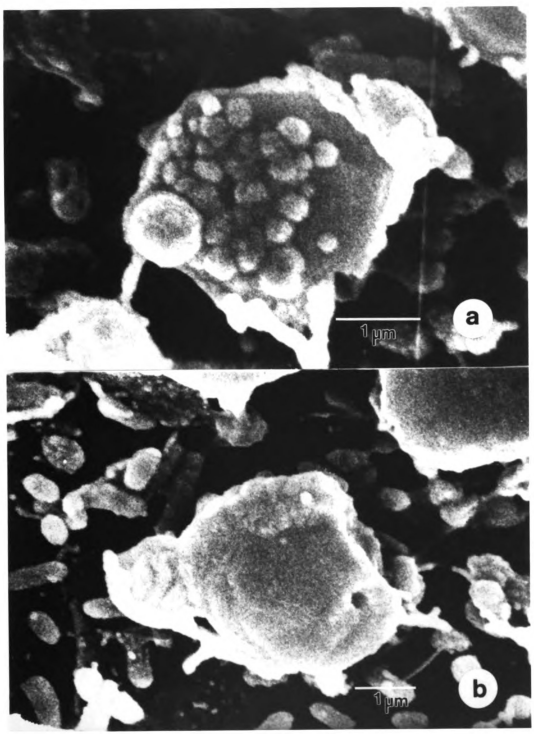

1 µm
a
1 µm
b

Table 2. Mean* mortality of five batches of two day old larvae of Ae. triseriatus assayed against a single application of Leptolegnia sp. inoculum

Larval batches	Mean number of larval inoculated	Mean** larval mortality per assay time (hours)		
		24	48	72
first batch	500	455.50 +/-17.04	499.00 +/-1.20	500.00 +/-0.00
second batch	500	419.25 +/-63.53	494.00 +/-8.76	499.00 +/-2.00
third batch	500	361.50 +/-88.32	474.00 +/-32.78	492.50 +/-8.70
fourth batch	500	317.25 +/-60.10	473.25 +/-31.14	490.25 +/-12.82
fifth batch	500	334.00 +/-46.83	457.75 +/-39.86	486.50 +/-10.47

*Mean of 4 replications, 500 larvae per replication
**Mean +/- std. dev.

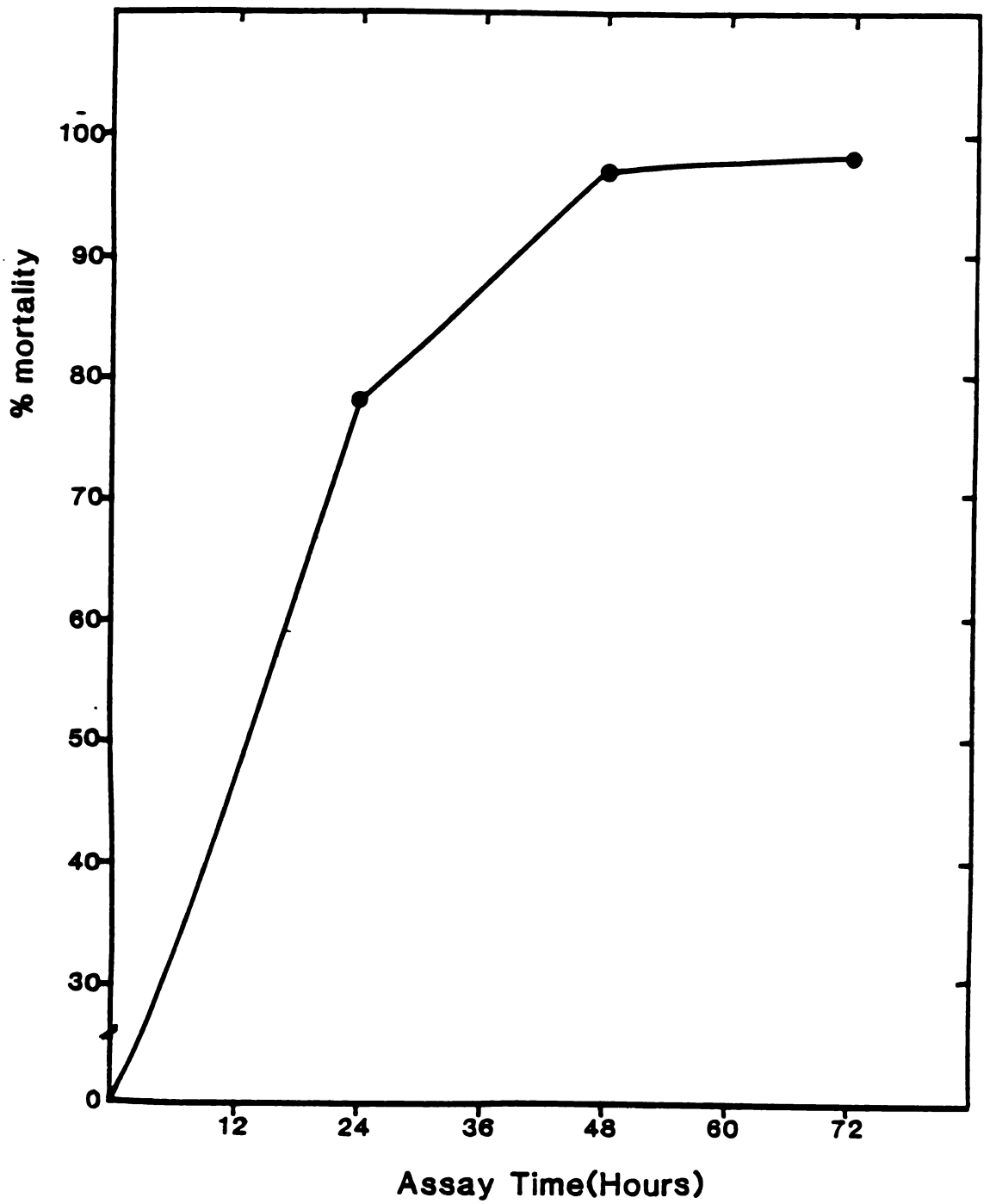
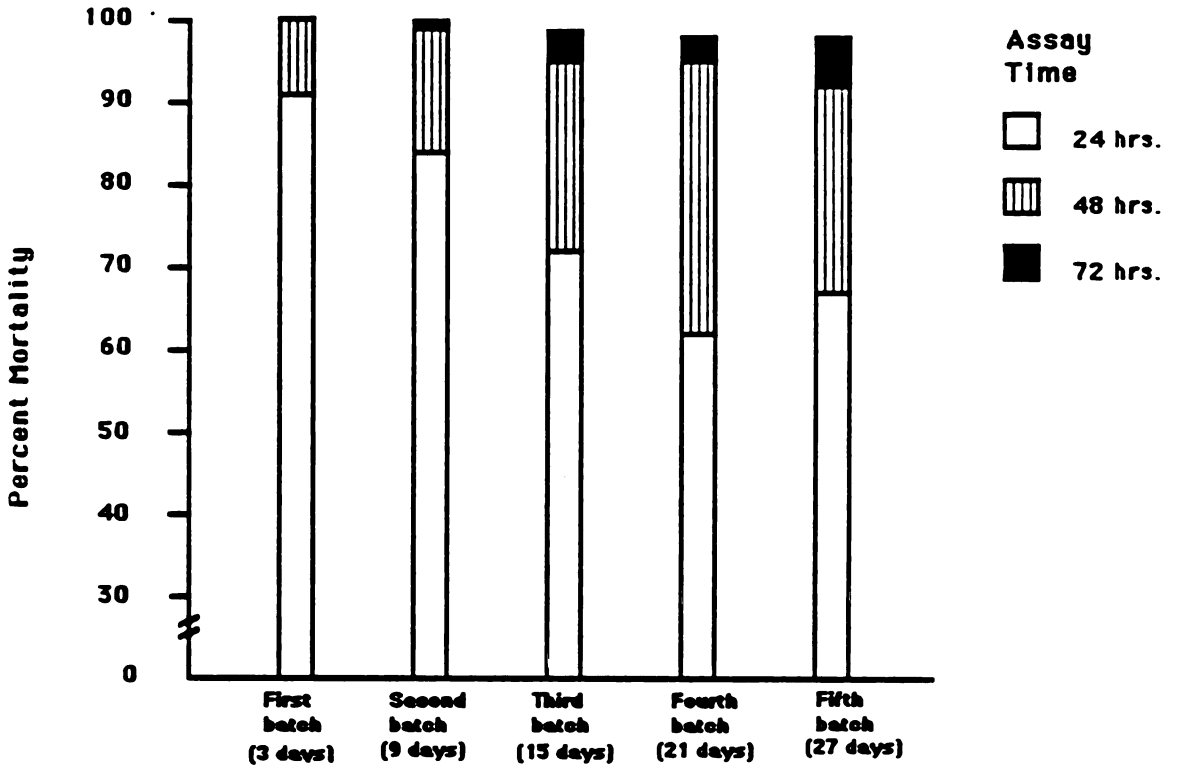


Figure 6. Average mortality rate of five batches of Ae. triseriatus larvae infected with a single application of Leptolegnia sp. inoculum.

An analysis of each batch indicated that the highest mortality occurred within 24 hours (Figure 7). The freshly prepared inoculation unit, applied to the first batch of the mosquito larvae was faster acting, killing 91% of the larvae within 24 hours and 9% more by the 48^{th} hour, bringing the total mortality rate at the 48 hour period to 100%. With subsequent batches, the pathogenic rate slightly decreased within the 24 hour period while slightly increasing for the 48 and 72 hour periods. In the second batch, 84% mortality occurred within 24 hours, 99% within 48 hours and 100% within 72 hours. The mortality from the 24^{th} to the 48^{th} hour was 15% and for the 48^{th} to the 72^{nd} hour was 1%. For the third batch 72% mortality occurred by the 24 hour, 95% at 48 hours and 99% at 72 hours. This indicated an actual mortality of 23% between 24 and 48 hour and 4% between 48 and 72 hour periods. Mortality in the fourth batch stood at 64% within 24 hours, 95% within 48 hours and 98% within 72 hours, indicating the actual number dead to be 31% between the 24^{th} and 48^{th} hour and 3% between 48^{th} and 72^{nd} hour. In the fifth batch, 67% mortality occurred within 24 hours, 92% within 48 hours and 98% within 72 hours. The actual death rate occurring between the 24^{th} and the 48^{th} hour was 25% and between the 48^{th} and 72^{nd} hour was 6%.



Time following activation of the inoculation unit

Figure 7. Persistent infection of five batches of Ae. triseriatus larvae by Leptolegnia sp.

Slight fluctuations occurred in the killing rate of the inoculation unit between larval batches introduced (Table 3). During the first 24 hours after exposure of the host to the pathogen the mortality rate decreased from the first through the fourth batch but increased by 3.94% for the fifth batch. At the 48^{th} hour period mortality had increased in all batches until the fifth, where there was a decrease of 6.38%. At 48 hours, most larvae had been killed. Between the 48^{th} and the 72^{nd} hour the mortality rate of the remaining larvae increased with each successive batch except for the fourth. In that batch, the mortality rate was less than in either the third or the fifth batch (Table 3). There was no significant difference for all batches in either the rate or potential for pathogenicity during the whole twenty-seven days of testing, as determined by Duncan's multiple range test at 0.05 confidence level using the general linear model of SAS statistical procedure.

C. Orientation of the Zoospores of *Leptolegnia sp.* Towards the Larvae of *Ae. triseriatus*

The orientation experiment tested the ability of motile zoospores to encyst and adhere on a preferred target prior to germination and subsequent penetration into that target. Counts of the encysted zoospores on the three given targets showed that more zoospores encysted and

adhered on tubes that contained the larval homogenate than on either the tubes that contained the plain agar or were empty (void of solid medium but containing water that entered from the petri dish) (Table 4). Seventy-two percent of the zoospores encysted and adhered to the tubes that contained larval homogenate, but tubes with plain agar had 21% encystment and empty tubes had 7% encystment.

Table 3. Differences in mortality of larvae infected by the same inoculation unit with each succeeding batch.

Batches Compared Assayed	Differences in Larval Mortality (%) Between Batches per Time (hours) 24	48	72
First and second batch	7.08 (-)	6.28 (+)	0.80 (+)
Second and third batch	10.62 (-)	7.86 (+)	2.76 (+)
Third and fourth batch	8.69 (-)	8.98 (+)	0.29 (-)
Fourth and fifth batch	3.94 (+)	6.38 (-)	2.44 (+)

(+) indicates increase between the two batches
(-) indicates decrease between the two batches

Table 4. Number of zoospores that swam to, encysted and adhered to tubes (mean +/- SD)*

Treatment	Ends**	Center	Total
Homogenate	1598.0+/-68.41[a]	5037.5+/-421.0[a]	8233.5 +/-437.4[a]
Plain Agar	542.8+/-82.05[b]	1344.2+/-169.65[b]	2429.75+/-324.3[b]
Empty	46.0+/- 3.24[c]	718.0+/- 70.74[c]	810.0 +/- 77.03[c]

*Means followed by different letters within a column are different at the .01 level as determined by LSD test on data transformed with LogX.

**Means of two ends of the tubes.

A significant number of zoospores encysted on tubes that contain media compared to empty tubes. Fifty-one percent more zoospores encysted on tubes with homogenate media compared to tubes with plain agar. A comparison of the number of encysted zoospores collected from the ends and middle of the tubes indicated encystment in each treatment occurred more in the center of the tubes than on the two ends combined. The tubes with homogenate had 61% encystment on the center compared to 39% on the ends; the plain agar had 55% on the center compared to 44% on the ends; and the empty tube had 89% on the center and 11% on the ends. Figure 2 is a diagrammatic presentation of the tubes and encysted zoospores.

D. Mode of Infection of *Ae. triseriatus* Larvae by the Zoospores of *Leptolegnia sp.*

It was observed during all experiments that the mosquito larvae rushed to the fungal inoculation units as they were introduced into the infection bowls and jars. Many larvae clustered around the units, moving their mouthparts very rapidly, and some larvae started actively gnawing at the units. Some of these larvae became entangled in the mycelia of the inoculation unit during the rush to gnaw and eventually died entangled there. The larvae that first clustered around the inoculation unit became moribund much sooner than those that visited the unit later. Diagnostic microscopic examination of all the dead larvae for symptoms of leptolegnial infection revealed the presence of zoospores and encysted zoospores in the hemocoel of the dead larvae. The developmental stage of the zoospores found in the hemocoel varied at various time intervals between inoculation and examination. Microscopic examination of larvae inoculated with fungal units that were stained with crystal violet revealed many violet colored particles in the digestive tract, more pronounced in the regions of the foreguts and midguts.

The larvae that were examined six hours after exposure to the pathogen showed engorgement at the esophagus and proventricular regions. Encysted zoospores were lodged

between the teeth of the larval proventromentum (Figure 8). There was also increased palpitation in the whole gut region. The larvae that were examined eleven to twelve hours after exposure had zoospores swimming in the hemocoel and an increase in the number of hemocytes in the hemocoel. The midgut and the anal areas were highly distended. Uninfected larvae generally showed normal rhythmic muscle contractions and lacked spore-like structures in the hemocoel (Figure 9A), but the infected moribund larvae showed excessive contractions of the muscles in the gut and head regions. Most of those moribund larvae had encysted spores in the hemocoel near the lobes of the gastric caeca (Figure 9B).

Twelve to fifteen hours after exposure there were many encysted zoospores in the hemocoel of the thoracic, abdominal and anal regions, inside the anal papilla, digestive tract and trachea of the larvae. In the thoracic region, a number of encysted spores were lodged in the esophagus (Figure 10) while others had descended to the anterior end of the crop (Figure 11). In the abdominal region, encysted zoospores were found in the midgut (Figure 12). Masses of encysted primary and secondary zoospores were found in the tracheal matrix (Figure 13). Wound spots were often seen on the walls of the trachea and midgut

Figure 8. Photomicrograph of encysted zoospores (arrows) of Leptolegnia sp. lodged between the teeth of the proventromentum of Ae. triseriatus larva. Six hours after exposure.

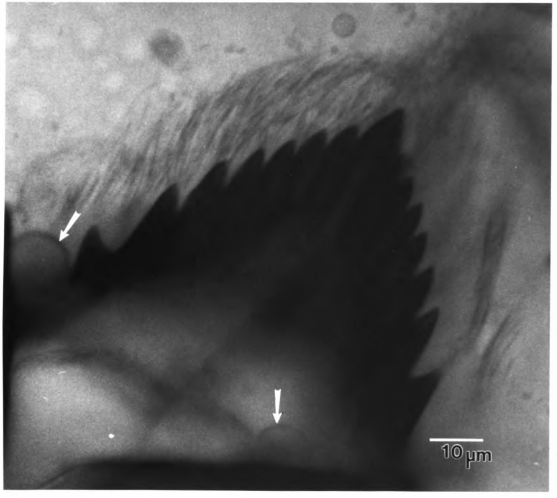

10 µm

Figure 9. Photomicrographs of the gastric caeca with surrounding hemocoel in uninfected and infected larvae of Ae. triseriatus.

A. Normal appearance of the gastric caecum showing lack of spores in the hemocoel area.
B. Abnormal appearance of gastric caecum in infected, moribund larva for 12 hours after exposure. Note the presence of encysted zoospores (arrows) of Leptolegnia sp. in the surrounding hemocoel.

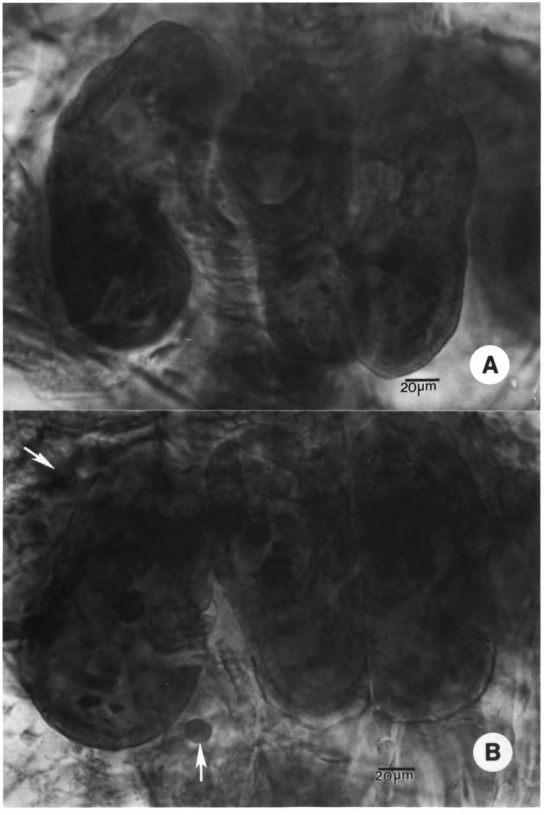

20µm
A
B
20µm

Figure 10. Photomicrograph of encysted zoospores (arrow) of Leptolegnia sp. in the esophagus of the larva of Ae. triseriatus twelve hours after exposure.

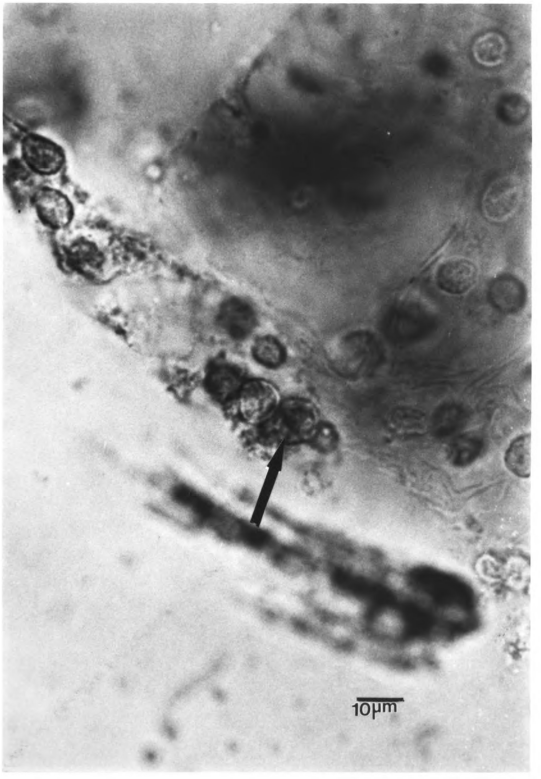

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Figure 11. Photomicrograph of encysted zoospores (arrow) of *Leptolegnia sp.* in the crop of *Ae. triseriatus* larva. Twelve hours after exposure.

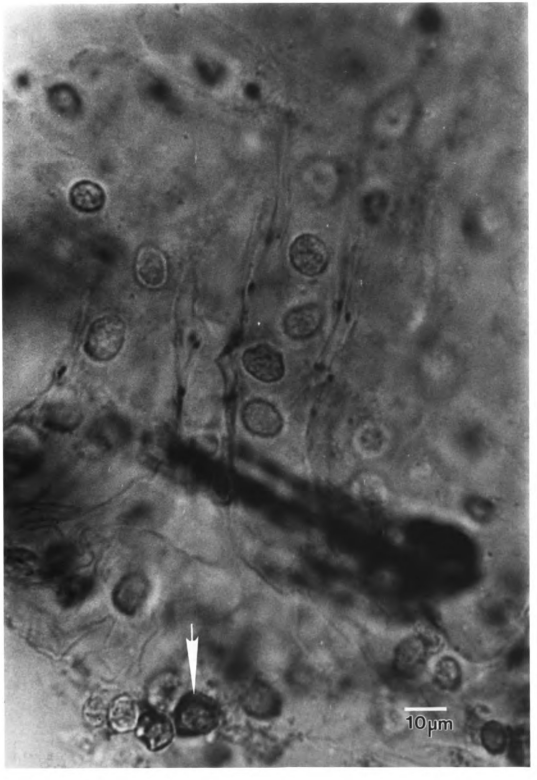

10µm

Figure 12. Photomicrograph of encysted zoospores (arrows) of *Leptolegnia sp.* in the midgut of *Ae. triseriatus* larva. Fifteen hours after exposure.

pz denotes primary encysted zoospore
sz denotes secondary encysted zoospore
t denotes the trachea

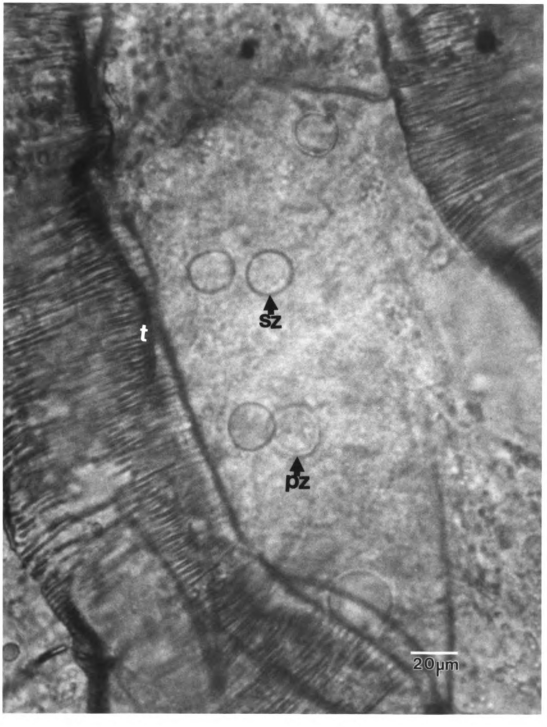
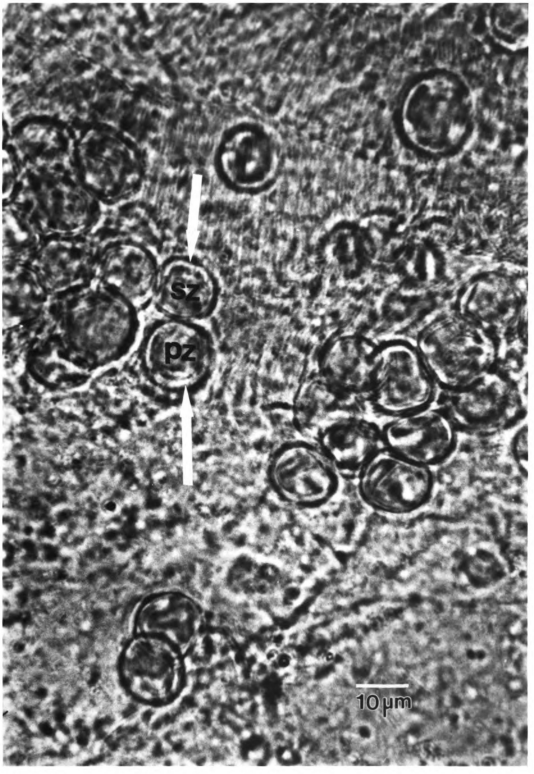

t
sz
pz
20 µm

Figure 13. Photomicrograph of primary (pz) and secondary (sz) encysted zoospores (arrows) in the trachea of the abdominal region of Ae. triseriatus larva. Fifteen hours after exposure.

The spiral striations on the background denote the taenidia of the trachea.


sz
pz
10 µm

where encysted spores in both organs were in close proximity.

In the abdominal hemocoel, both primary and secondary encysted spores were observed in close contact with the fat body which they often destroyed, leaving grey spheres with their surrounding lamina, while the hemocytes challenged and often destroyed some of the encysted spores, leaving dark spheres (Figure 14). Encysted zoospores (Figure 15A) were also seen on the integument of the siphon tube, and in the hemocoel within the siphon tube where they either germinated or became cluttered by surrounding hemocytes (Figure 15B). Zoospores also encysted on the ventral brush (Figure 16A) and on the integument of the anal papillae (Figure 16B), abdomen and thorax. It was observed that some spores encysting in the anal papilla, germinated within fifteen hours while those encysting on the integument did not. Also, the spores that encysted on the integument of any region of the larvae often did not adhere through the washes, fixation and dehydration procedures used in preparing the specimen for photomicroscopy.

Twenty-four hours after exposure, numerous spores were in the hemocoel (Figure 17). The midgut, hindgut and rectum of the larvae were extremely engorged with rod shaped bacteria; some of these were moving in the hemocoel and trachea (Figure 18). A wound spot (Figure 18) was seen

Figure 14. Photomicrograph of encysted zoospores (e) of Leptolegnia sp. in the hemocoel (ce) of Ae. triseriatus larva. Fifteen hours after exposure.

Normal fat body (f) appears clear with surrounding lamina, but fat body destroyed by the zoospore appears gray (d) with surrounding lamina. (c) denotes darkened spheres of encysted zoospores destroyed by the hemocyte (h).

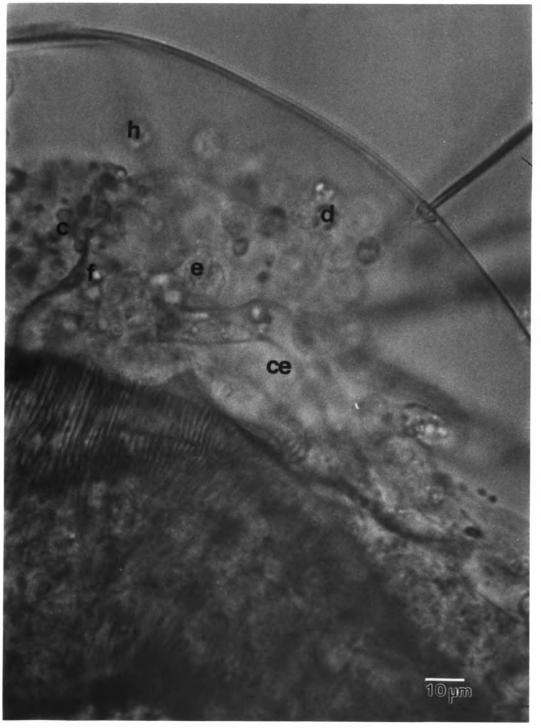

h
c
d
f
e
ce
10µm

Figure 15. Photomicrograph of encysted zoospores (e) of *Leptolegnia sp.* in the larvae of *Ae. triseriatus*.

A. On the integument of the siphon tube
B. In the hemocoel of the siphon tube cluttered by surrounding hemocytes (h).

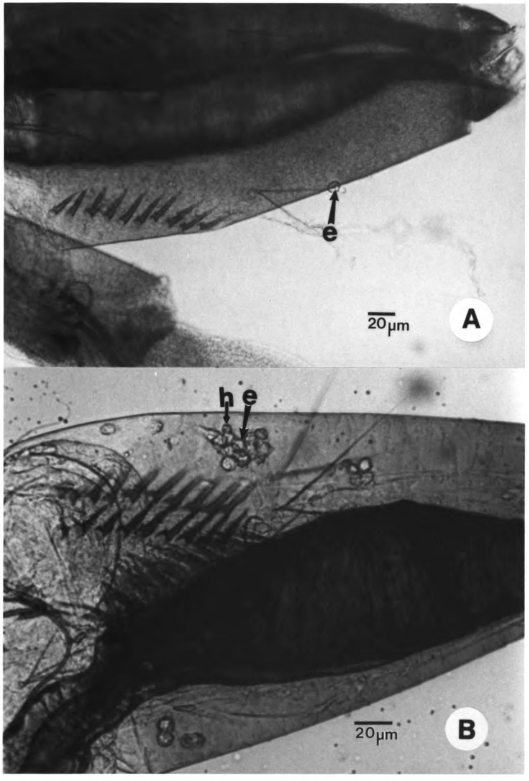

e
20µm
A
h
e
20µm
B

Figure 16. Photomicrograph of encysted zoospores (arrow) of *Leptolegnia sp.* on the larvae of *Ae. triseriatus*. Fifteen hours after exposure.

A. On the ventral brush
B. On the integument of the anal papilla

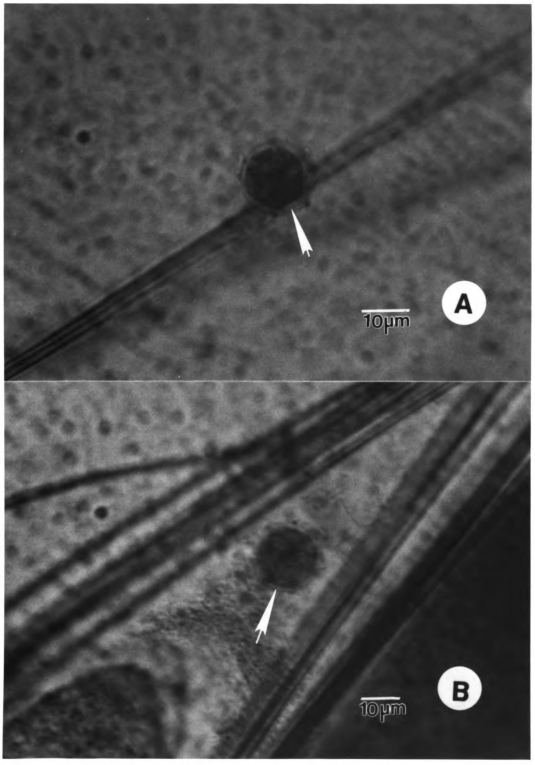
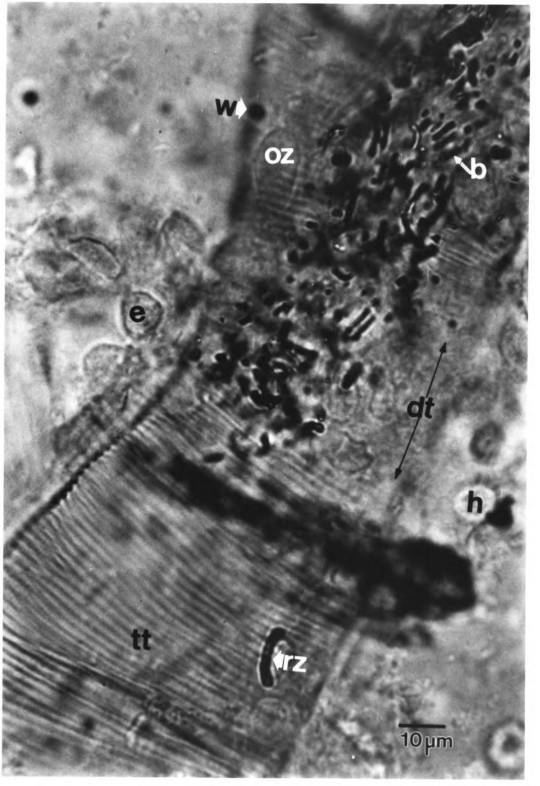

10μm
A
10μm
B

Figure 17. Scanning electron micrograph showing encysted zoospores (e) of *Leptolegnia sp.* in the hemocoel of *Ae. triseriatus* larvae. Twenty-four hours after exposure. Cryofracturing technique was used to expose the hemocoel.


e
10 µm

Figure 18. Photomicrograph of zoospores in association with mass of bacteria in the trachea of Ae. triseriatus larva infected with Leptolegnia sp. Twenty-four hours after exposure.

(W) denotes a wound spot, site of zoospore entrance into the trachea. Note an oval-shaped motile zoospore (oz) adjacent to the wound spot. Rod-shaped zoospore (rz) often associated with bacteria (b) were also present in the tracheal matrix whose taenidia (tt) were often destroyed leaving a collapsed region (dt). Encysted zoospores (e) were found in the adjacent gut. (h) denotes hemocyte that had been damaged leaving clear lysed sphere.


w
oz
b
e
dt
h
tt
rz
10µm

on the wall of the trachea, denoting a site of penetration by the zoospore. An oval shaped zoospore (Figure 18) was inside the trachea adjacent to the wound mark, and a rod-shaped zoospore (Figure 18) was also seen inside the trachea. Other configuration of zoospores were also present in the trachea and adjacent gut and hemocoel (Figure 18). A section of the trachea and a hemocyte in adjacent area of the hemocoel were already damaged (Figure 18), while healthy hemocytes attempted to engulf the bacteria or zoospores. Some spores in the visceral organs of the larvae germinated, and developed hyphae that grew either longitudinally within the hemocoel or vertically towards the integument or an adjacent organ. The hyphae that grew towards the integument pierced through the larval integument (Figures 19A and 19B) within 24 hours, while any spore encysted on the integument did not germinate in that location but either remained in that state on the integument or fell off and germinated in the water medium.

Three different phenomena occurred within forty-eight hours after exposure to the pathogen. SEM micrograph of uninfected and infected larvae after this hour showed, in uninfected larvae, normal projecting hairs and brushes, characteristics of the mosquito larvae (Figure 20A), but in infected larvae, masses of hyphae (mycelia) projected from various regions of the larval body (Figure 20B). These

Figure 19. Photomicrograph of:

A. A hypha of Leptolegnia sp. growing from the visceral organ outward through the integument (li) of Ae. triseriatus larva. Twenty-four hours after exposure.

B. An enlargement of the marked section of (A) to show the direction of the hyphal growth from the hemocoel (ce) and the protrusion of the hypha (ha) through larval integument (li).

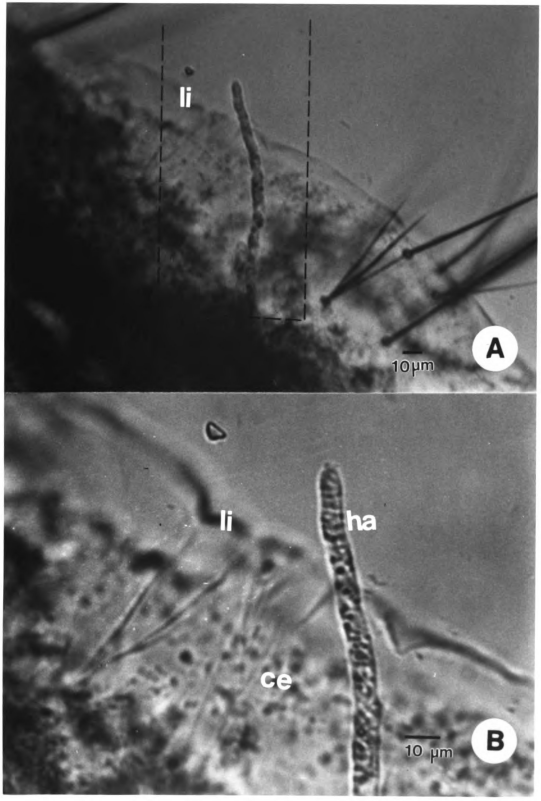
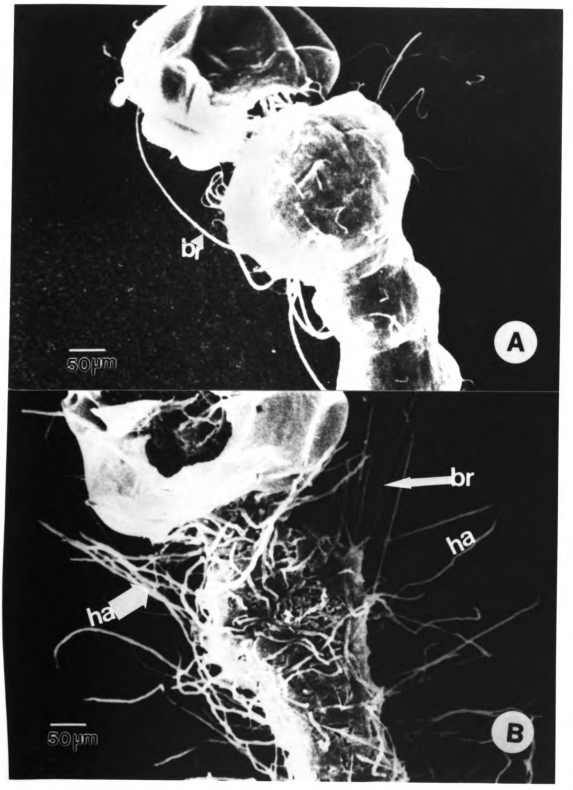

li
10µm
A
li
ha
ce
10 µm
B

Figure 20. Scanning electron micrograph of:

A. Uninfected larva of Ae. triseriatus showing normal projecting hairs and brush (br). Forty-eight hours control.

B. The mass of hyphae (ha) of Leptolegnia sp. projecting from the body of Ae. triseriatus larva. Forty-eight hours after exposure. Note mycelia within the hemocoel through cracked larval integument.


br
50μm
A
br
ha
ha
50μm
B

hyphae were quite distinct from the characteristic hairs and brushes. The hemocoel of such infected larvae were partially filled with mycelia and sporangia. Light microscope examination of some of the stained infected larvae showed mycelial mat forming on the integument while some of the hyphae project out (Figure 20C).

Some infected larvae that did not have mycelial development showed some normal encysted spores and melanized, encysted zoospores in the hemocoel (Figure 21A). Some of the sporangia that had formed became melanized within the hemocoel (Figure 21B). Sporangial melanization might be partial, covering only the tip of the sporangium, while the rest of the sporangium remained normal, or the entire length of the sporangium might be melanized (Figure 21B). Some encysted spores were partially engulfed by the hemocytes, while others were partially destroyed or disfigured (Figure 21B). The third observed phenomenon was the presence of encysted zoospores in the antenna (Figure 22).

Seventy-two hours after exposure the outside of the larval body was completely covered by a mass of mycelia (Figure 23A) and the hemocoel was completely filled with fungal mycelia. Most of these mycelia had transformed into sporangia and some zoospores had been produced at this

Figure 20C. Photomicrograph of infected Ae. triseriatus larva showing mass of mycelia (m) covering the larval body, and some hyphae (ha) growing out distinctly different from the characteristic hair and brushes (br).

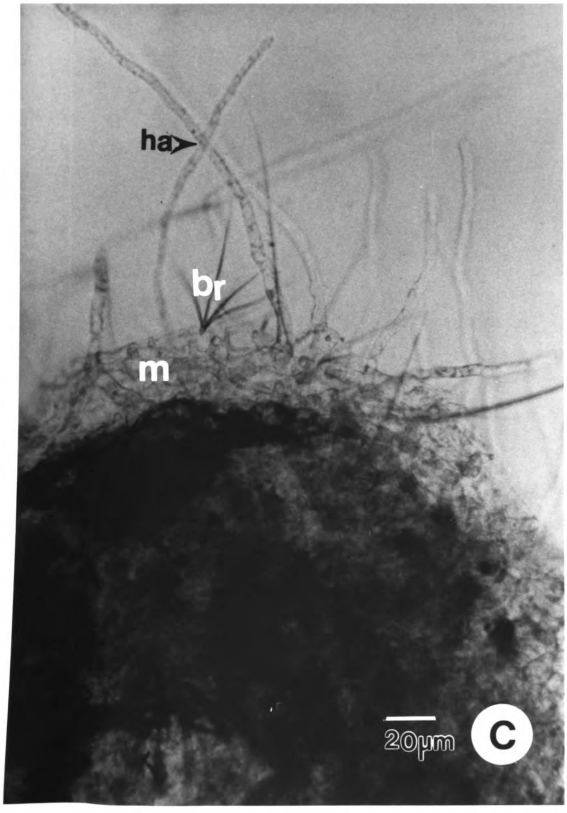

ha
br
m
20µm
C

Figure 21. Photomicrograph of:

A. Melanized encysted zoospores (me) and normal encysted zoospores (ne) in the hemocoel of Ae. triseriatus larva. Forty-eight hours after exposure.

B. Melanized sporangia (ms) of Leptolegnia sp. in the hemocoel of Ae. triseriatus larva. Forty-eight hours after exposure.

Note a section of the sporangia remained normal (k) while the anterior segment of the same sporangia became melanized (y). Hemocytes often attacked encysted zoospore by coupling to it (ax) while some of the encysted zoospore became partially destroyed or disrupted (pe).

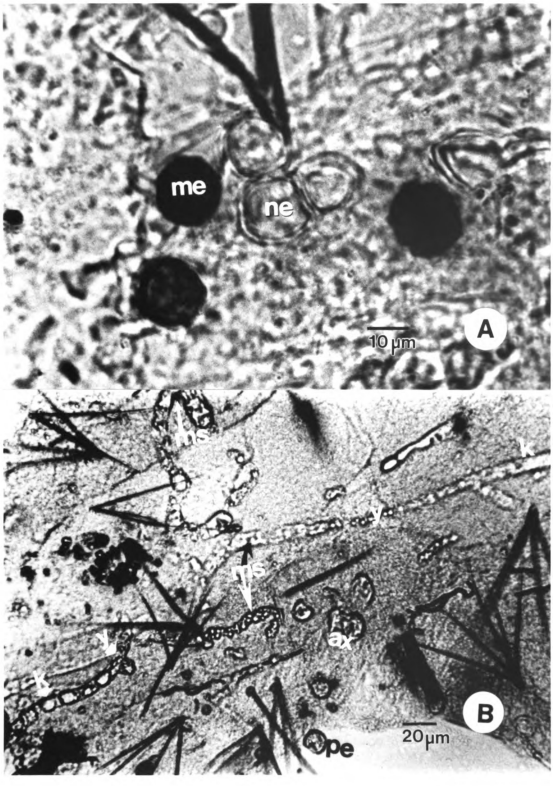
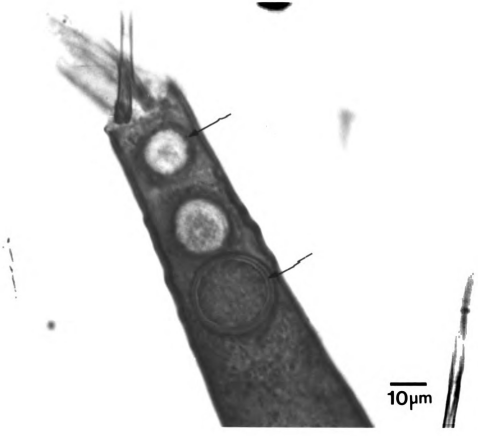

me
ne
10 µm
A
ps
ps
ax
B
20µm
pe

Figure 22. Photomicrograph of encysted zoospores of *Leptolegnia sp.* in the antennae of the larvae of *Ae. triseriatus*. Forty-eight hours after exposure.


10µm

time, while a few spores that had encysted on the integument had germinated (Figure 23B).

E. Effect of Starvation on the Susceptibility of the Larvae of Ae. triseriatus to Infection by Zoospores of Leptolegnia sp.

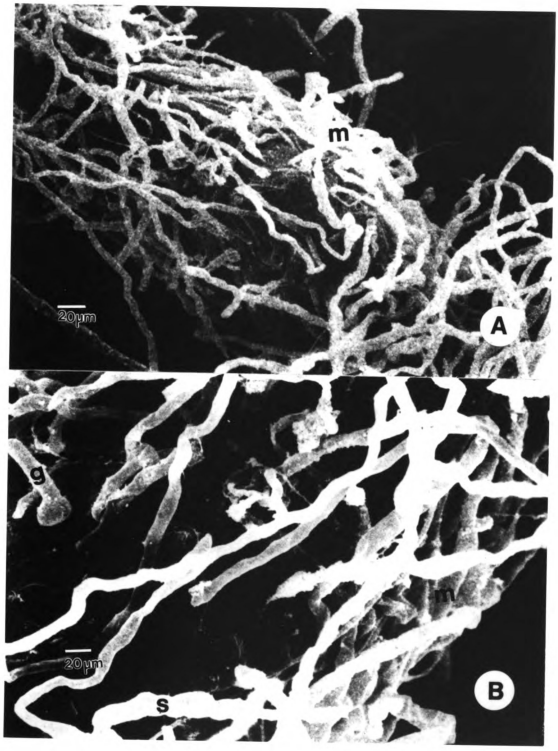
Two-day old larvae of Ae. triseriatus, starved prior to exposure to Leptolegnia sp., suffered a 100% mortality within 48 hours, with 87% of the larvae being killed within 24 hours of exposure. A mortality curve was plotted to illustrate the rate of susceptibility as an index of pathogenicity (Figure 24). There was no statistically significant difference in the overall susceptibility of fed and unfed larvae to infection, since both populations suffered 100% mortality within 48 hours of exposure (Figure 25). There was, however, a slight difference in the rate of susceptibility between starved and fed larvae in the first 24 hours post exposure. While 91% of the fed larvae died within 24 hours, only 81% of the starved larvae died within that same time period.

The second part of this experiment involved exposure of the starved larvae to the pathogen for 24 hours and 72 hours, respectively. The result indicated that the length of exposure time had no significant effect on the susceptibility of the starved larvae to infection by the pathogen. The fed larvae and starved larvae that were

Figure 23. Scanning electron micrograph of:

A. Leptolegnia sp. on the body of Ae. triseriatus larva. Seventy-two hours after exposure.

B. An enlargement of 23A. to show the germinated zoospore (g) the sporangia (s) that have developed, and the mycelial mat (m) covering the larval body.


m
20µm
A
g
n
20µm
B
s

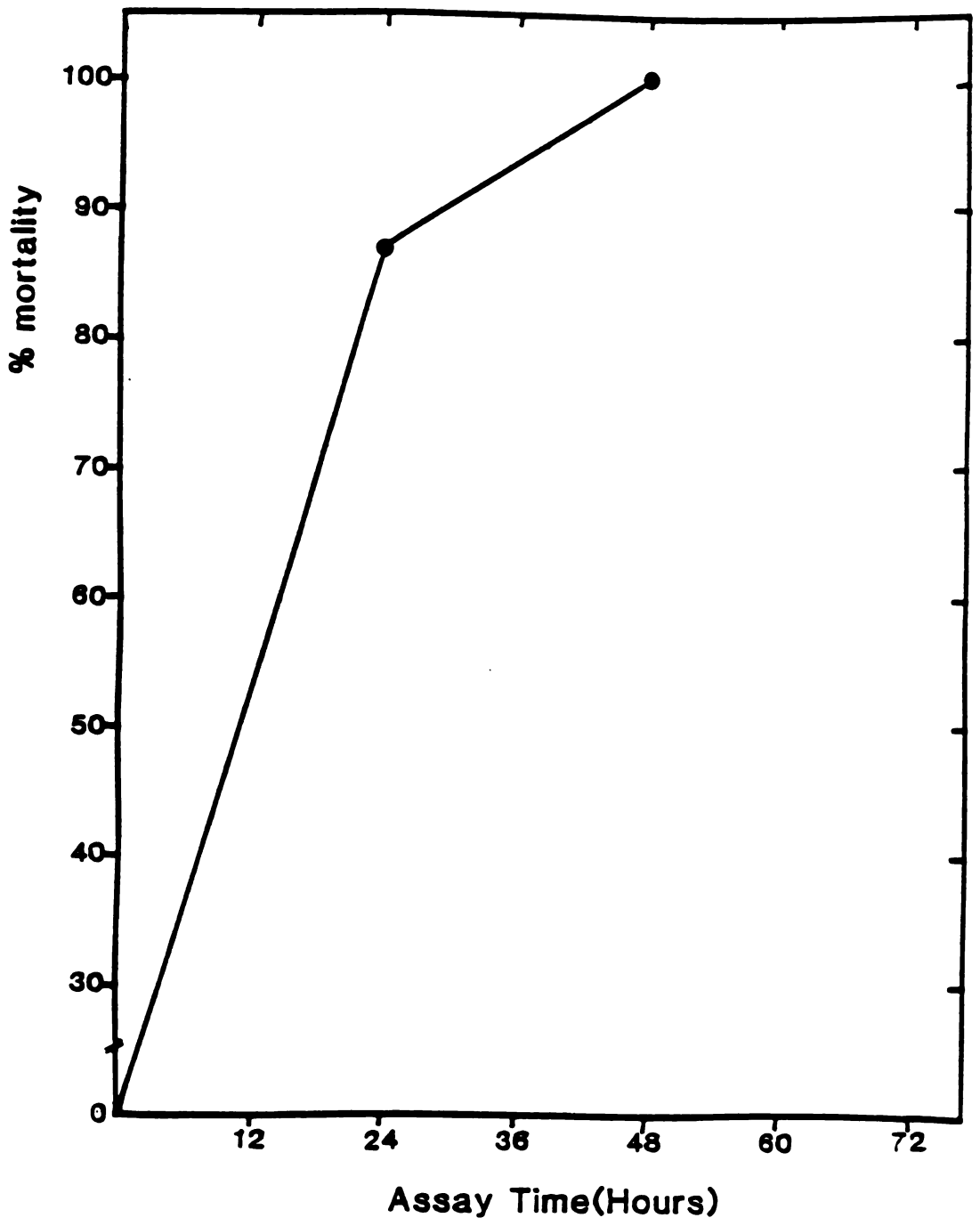


Figure 24. Mortality rate of starved two-day old larvae of Ae. triseriatus infected by zoospores of Leptolegnia sp.

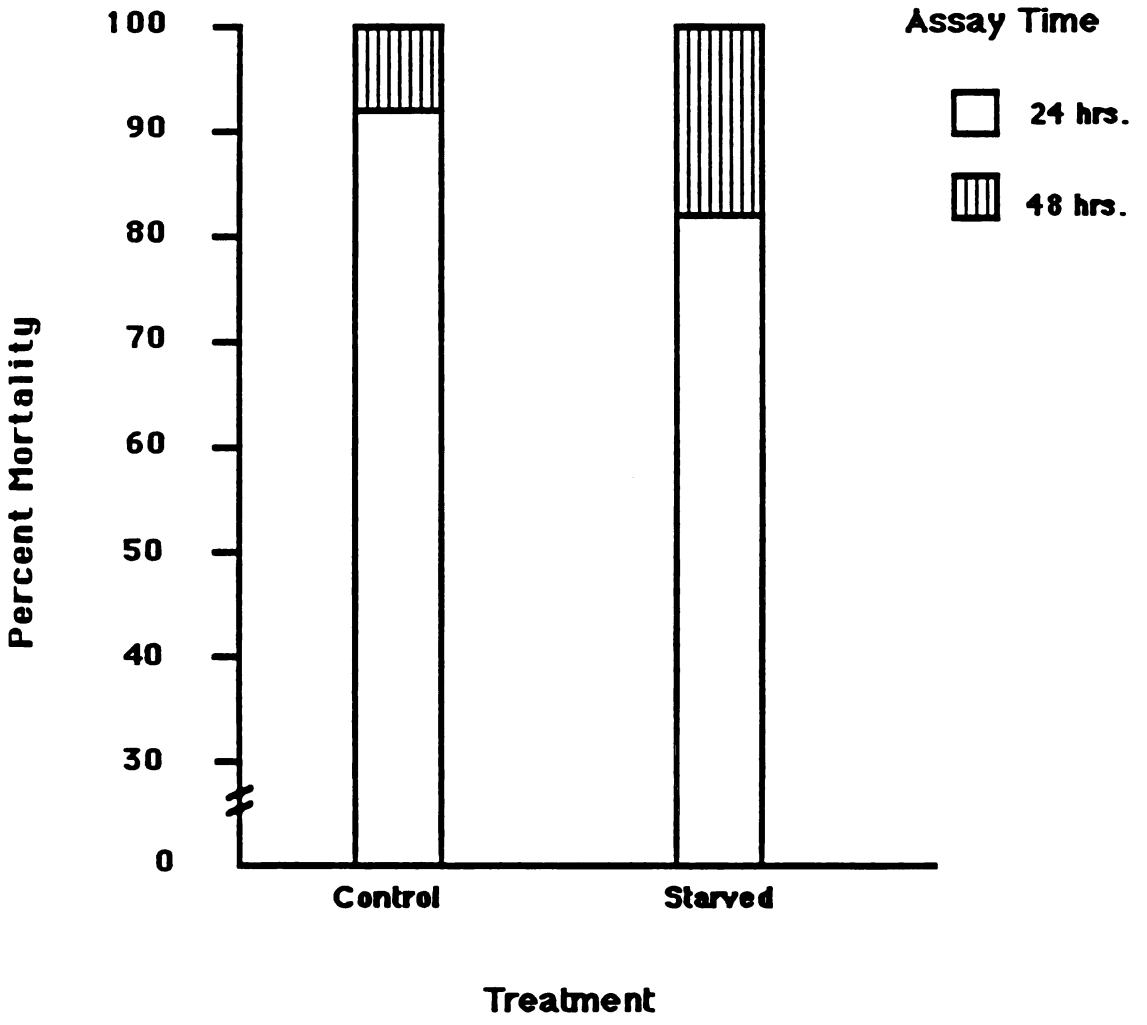


Figure 25. Effect of starvation on the susceptibility of Ae. triseriatus larvae to infection by Leptolegnia sp.

exposed to the pathogen for 24 hours, suffered 100% mortality within 48 hours. The starved larvae that were incubated with the pathogen for 72 hours suffered 99.4% mortality within 48 hours (Figure 26). The rate of susceptibility seemed to decrease not only with starvation but also with an increase in exposure time to the pathogen. Twenty-four hours after exposure, the fed larvae suffered 91% mortality, the starved larvae that were exposed to the pathogen for only 24 hours suffered 81% mortality while the larvae that were exposed for 72 hours suffered 71% mortality within the first 24 hours. As the susceptibility rate for the first 24 hours slightly decreased with starvation and exposure time, the susceptibility rate between the 24^{th} and the 48^{th} hour period slightly increased with starvation and increase in exposure time. Only 9% mortality occurred among the fed larvae between the 24^{th} and 48^{th} hour. The starved larvae that were exposed for 24 hours suffered 19% mortality between the 24^{th} and the 48^{th} hour period, while the starved larvae that were exposed for 72 hours suffered 28% mortality between the 24^{th} and 48^{th} hour period (Figure 26). There was no statistically significant difference in the pathogenic rate of Leptolegnia sp. on starved vs. fed larvae. The rate varied within each population in accordance with assay time but not in respect to starvation. All the larvae tested, both starved and

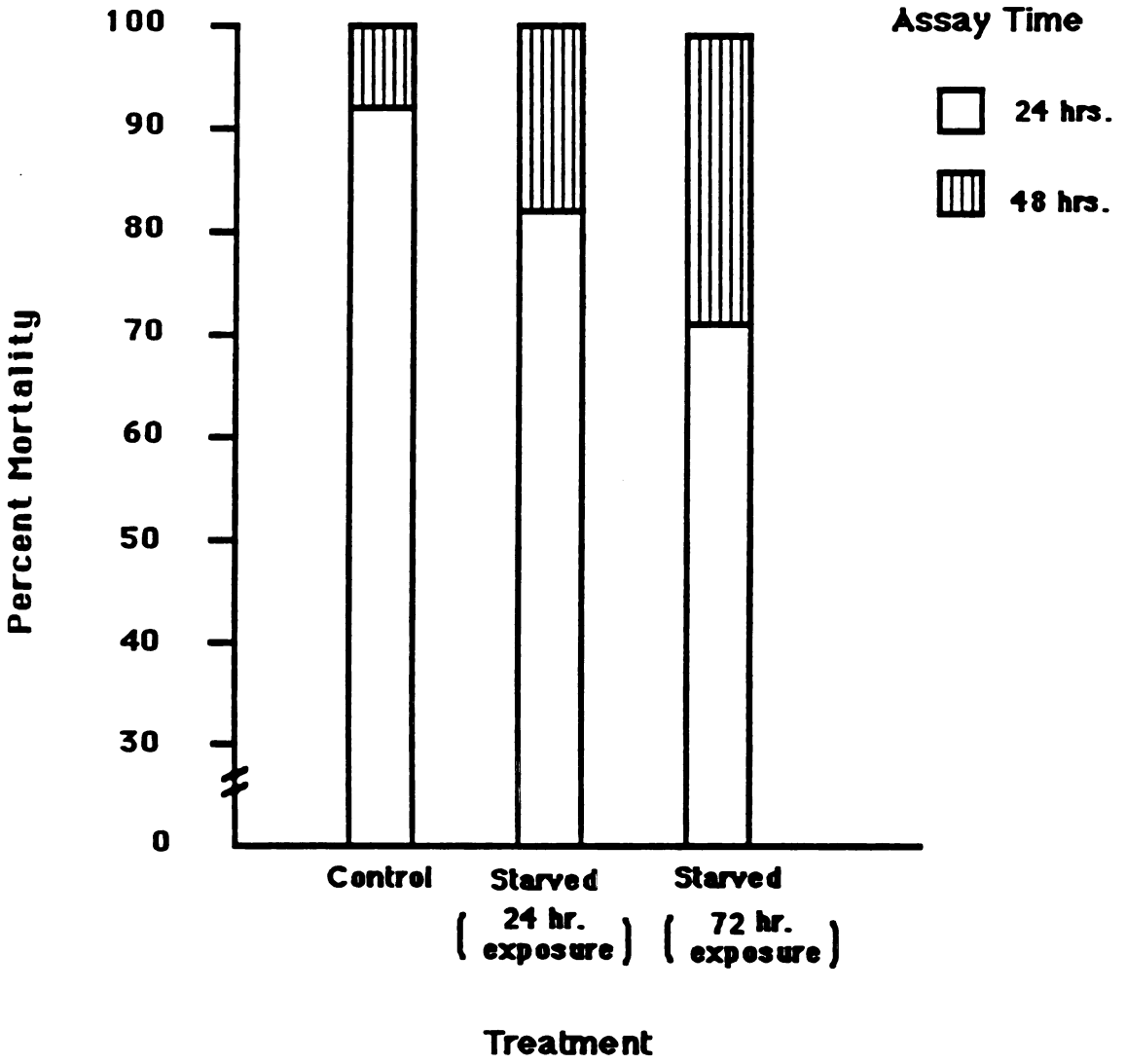


Figure 26. Effect of varied exposure time on the susceptibility of starved larvae of Ae. triseriatus to infection by Leptolegnia sp.

unstarved, were killed within 48 hours, and the rate of pathogenicity remained the same for all three groups.

F. Effect of Storage Temperatures and Storage Period on the Viability and Pathogenicity of Leptolegnia sp.

This two part experiment tested the ability of Leptolegnia sp., cultured in agar slants or established on split hempseed, to remain viable and pathogenic after a period of storage at selected temperatures. The slant cultures were stored at 5°C and 25°C for 24 months while the hempseed cultures were stored at -20°C, 0°C, 5°C and 25°C for 3, 6, 9 and 12 months. The result indicated that Leptolegnia sp. on half split hempseed, stored at 0°C, and -20°C would not remain viable for 3 months and the addition of 10% glycerol did not inhibit their loss of viability at these temperatures for that length of time (Table 5).

Table 5. Storage Temperature of Leptolegnia sp. Established on Half Split Hempseed

Temperature(oC)	Viability of Leptolegnia sp.
-20	-
0	-
5	+
25	+

- pathogen did not survive 0-3 months later
+ pathogen survived 0-12 months later

The viable Leptolegnia sp. established on half split hempseed was tested on a population of two day old larvae of Ae. triseriatus. The interactive effect of temperature, storage period and bioassay time on pathogenicity of stored inoculum in comparison with freshly prepared inoculum in infecting this aedian larvae was also examined (Table 6). The data obtained were analyzed to determine: (1) the mortality rate of Ae. triseriatus larvae infected with Leptolegnia sp. stored at 5°C and 25°C, respectively, for 3-12 months periods; (2) the effect of temperatures (5°C and 25°C), respectively, and storage periods of 3, 6, 9 and 12 months on the pathogenic potential of Leptolegnia sp. infecting Ae. triseriatus larvae; (3) the effect of storage temperatures (5°C and 25°C), respectively, and storage periods of 3, 6, 9 and 12 months on the pathogenic rate of Leptolegnia sp. infecting the larvae of Ae. triseriatus; and (4) the impact of bioassay time on the combined effect of temperature and storage period on the pathogenicity of stored Leptolegnia sp. towards the larvae of Ae. triseriatus.

Analysis on the effect of temperature indicated that Leptolegnia sp. stored at 5°C and 25°C for 3-12 months remained viable for the most part, with the exception of a few mycelia, and the viable ones did not loose their pathogenicity during the long period of storage. When

Table 6. Comparison of Mean* Mortality of Ae. triseriatus. Larvae Infected with Leptolegnia sp. Stored for 3, 6, 9 and 12 Months at Temperatures of 5°C and 25°C., and Freshly Prepared Leptolegnia sp. Larval Mortality Assayed at 24, 48, and 72 Hours After Inoculation.

Treatment Combinations	Mean** Larval Mortality per Assay Time (Hours) 24	48	72
Freshly prepared (control) Room Temperature	226.44+/-16.63	248.89+/- 3.67	250.00+/- 1.06
Stored 5°C for 3 months	215.75+/-18.84	243.75+/- 8.10	249.50+/- 1.00
Stored 5°C for 6 months	209.50+/-25.36	249.50+/- 0.58	250.00+/- 0.00
Stored 5°C for 9 months	171.25+/-16.86	240.75+/-10.63	249.25+/- 0.96
Stored 5°C for 12 months	146.75+/-19.19	198.50+/-32.89	225.75+/-19.62
Stored 25°C for 3 months	193.25+/-13.94	239.25+/-15.11	248.50+/-3.00
Stored 25°C for 6 months	158.50+/-46.08	227.00+/-16.99	243.50+/- 6.55
Stored 25°C for 9 months	94.25+/-50.23	199.25+/-22.87	233.50+/-14.73
Stored 25°C for 12 months	159.25+/-62.14	211.50+/-15.86	228.75+/-10.44

*Mean of 4 replications, 250 larvae per replication
**Mean +/- std.dev.

tested against two-day old larvae of Ae. triseriatus, both produced the expected mortality curve (Figure 27) with a slight difference in the rate. The Leptolegnia sp. stored at 5°C for 3-12 months induced 99% larval mortality within 72 hours while those stored at 25°C for 3-12 months induced a larval mortality of 96% within 72 hours. In the population of larvae exposed to Leptolegnia sp. stored at 5°C for 3-12 months, a mortality rate of 75% occurred within the first 24 hours, 19% within the second 24 hours, making a cummulative mortality of 94% for 48 hours; an additional 5% mortality occurred during the third 24 hours, resulting in 99% mortality for the 72 hour testing period. In the population of larvae exposed to Leptolegnia sp. stored at 25°C, a mortality rate of 61% occurred within the first 24 hours, 27% within the second 24 hours bringing the total death for 48 hours to 88%, and an additional 8% mortality occurred during the third 24 hours resulting in 96% mortality within 72 hours of the testing period (Figure 27).

In the second part of the analysis, where the effects of temperature and storage periods on pathogenic potentials are measured, the result indicated that there was no statistically significant difference in the pathogenic potential of Leptolegnia sp. stored at either 5°C or 25°C for 3 months. Both of them demonstrated the capability of

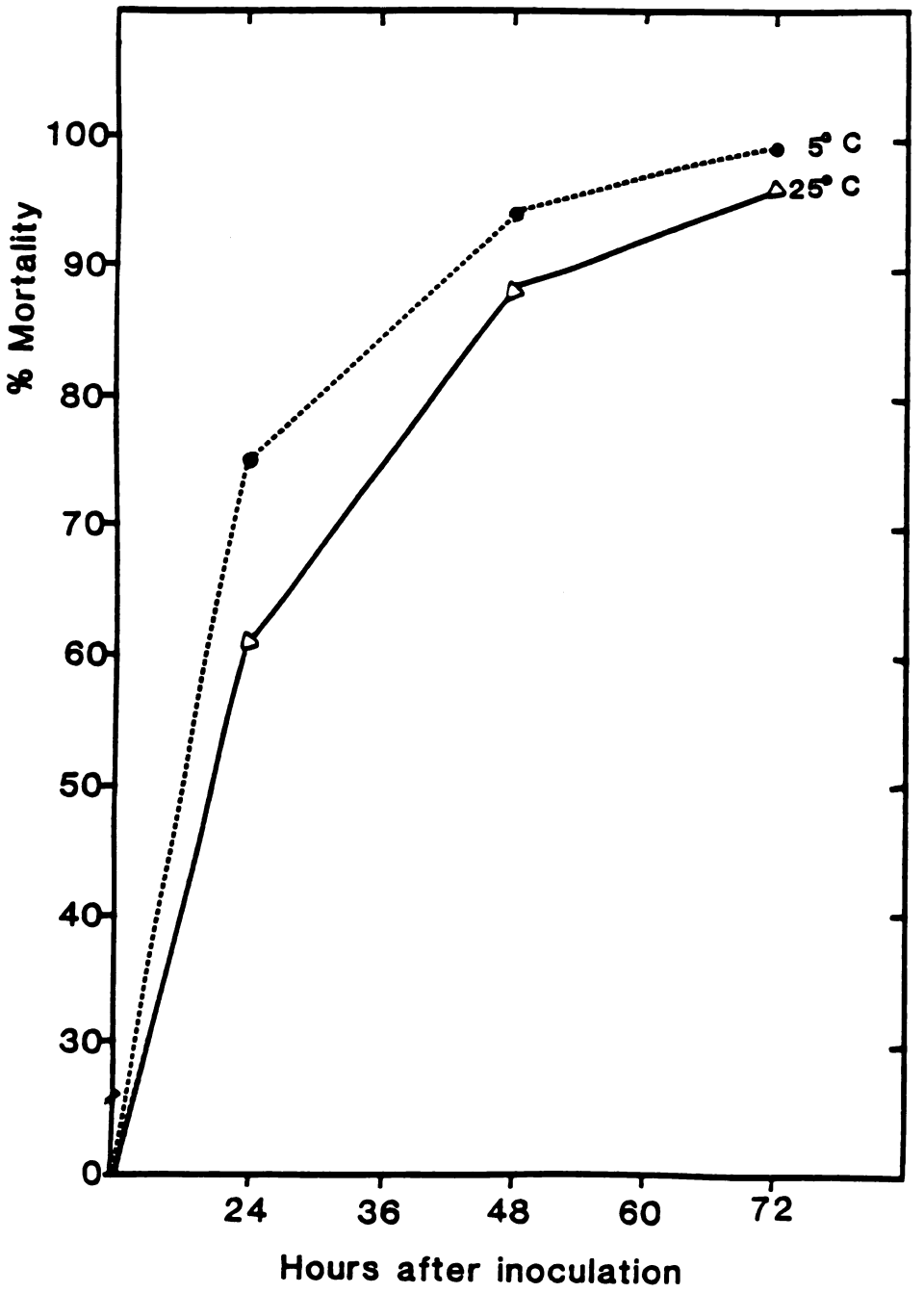


Figure 27. Mortality rate of two-day old larvae of Ae. triseriatus infected by zoospores of Leptolegnia sp. Stored at 5°C and 25°C for 3 to 12 months.

inducing 99% mortality after 3 months of storage, showing a mere 1% mortality lower than the control. There was only a minor difference of 3% between inocula stored at 5°C and those stored at 25°C in their potential to kill the host after 6 months in storage. Inoculum stored at 5°C showed the same potential as the freshly prepared inoculum, inducing 100% mortality in the larval host, while inoculum stored at 25°C induced 97% after a 6 months storage period. At 9 months of storage, the potential of inoculum stored at 5°C was 6% higher than that of the inoculum store at 25°C since the inoculum stored at 5°C induced 100% mortality while the one stored at 25°C induced 94% mortality in the host. There was no statistically significant difference between inoculum stored at 5°C and that stored at 25°C during 12 months in storage, since inoculum stored at 5°C demonstrated a killing potential of 92% while the one stored at 25°C killed 91% of its larval hosts. In comparison with the freshly prepared inoculum, inoculum stored at 5°C maintained a very high potential after 3, 6 and 9 months in storage but suddenly declined by 8% during the 9-12 month period, while the inoculum stored at 25°C showed a steady decline in its potential as the length of time in storage increased (Figure 28).

There were statistically significant differences in the pathogenic rate between freshly prepared Leptolegnia

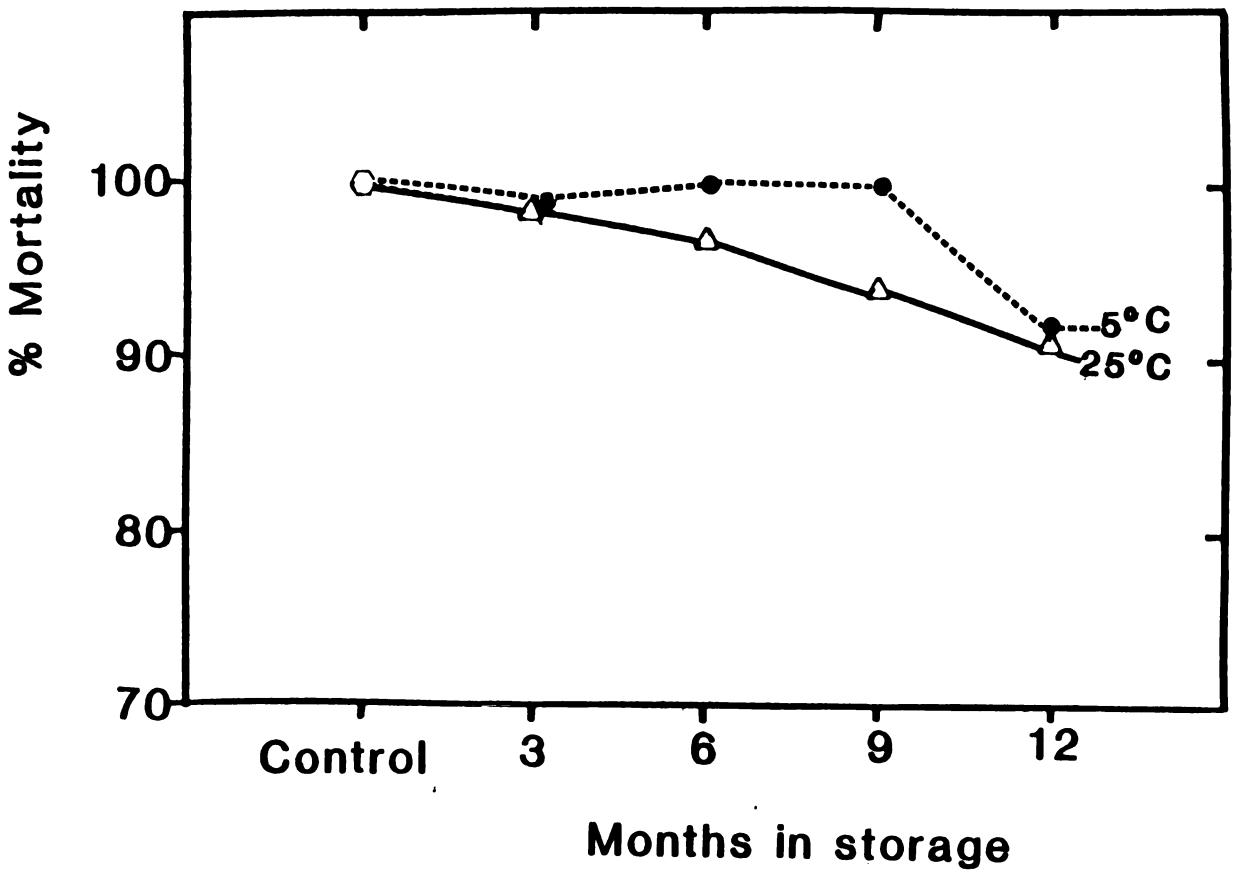


Figure 28. Effect of storage temperature (5°C and 25°C) on the pathogenic potential of Leptolegnia sp. Stored for 3, 6, 9 and 12 months prior to inoculating larvae of Ae. triseriatus.

sp. and Leptolegnia sp. stored at temperatures of 5°C and 25°C, for 3, 6, 9 and 12 months (Figure 29). Freshly prepared Leptolegnia sp. induced a 91% mortality level within 24 hours of exposure, and 9% additional mortality within the second 24 hours, bringing its total mortality to 100% within 48 hours. Leptolegnia sp. stored for 3 months at 5°C induced 86% mortality within the first 24 hours and those stored at 25°C induced only 77% mortality within that first 24 hours. During the second 24 hours, inoculum stored at 5°C induced additional 11% mortality bringing its mortality rate for 48 hours to 97% while inoculum stored at 25°C induced 18% mortality upgrading its rate within 48 hours to 95%. Both inoculum stored at 5°C and 25°C caused 99% mortality during the 72 hour period, the 5°C stored inoculum achieved this by killing 2% more larvae during the final 24 hour period while the 25°C stored inoculum attained the 99% level by killing an additional 4% larvae during the third 24 hour period. Exposure of larvae to inoculum stored for 6 months at 5°C caused larval mortality of 84% within the first 24 hours, and 16% from 24-48 hours resulting in a total mortality of 100% within 48 hours.

Inoculum stored at 25°C for 6 months caused 63% mortality within 24 hours, 27% from 24-48 hours increasing mortality at 48 hours to 90%, and further increasing it to 97% during the 72 hour period by killing 7% more larvae

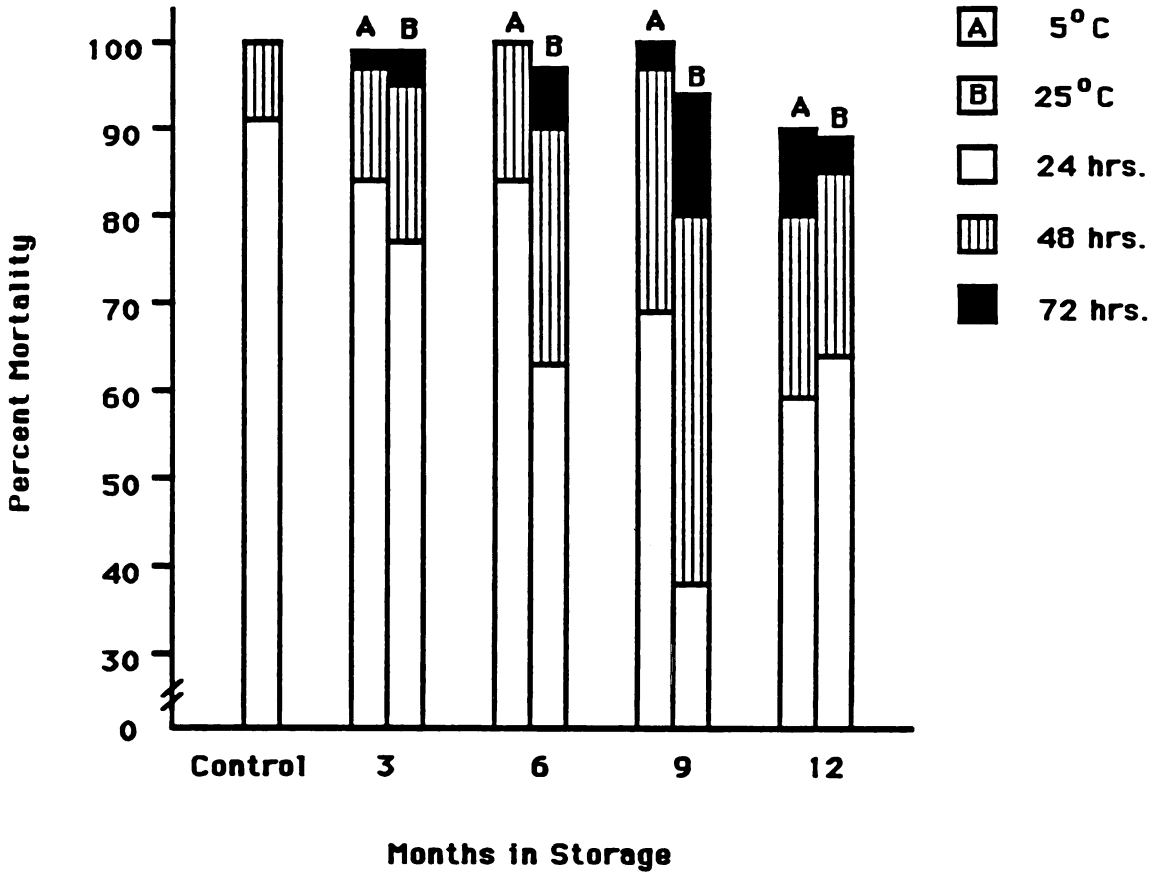


Figure 29. A comparison of mortality rate of two-day old larvae of Aedes triseriatus infected with zoospores of Leptolegnia sp. stored at 5°C and 25°C for 3, 6, 9 and 12 months (a measure of pathogenic rate).

from 48-72 hours. A very erratic mortality rate occurred after the 9 months storage period. Larvae exposed to the pathogen stored at 5°C for 9 months suffered 69% mortality within 24 hours, 28% within 24-48 hours, resulting in a total of 97% within 48 hours. An additional mortality rate of 3% from 48-72 hours brought the total mortality for the 72 hour testing period to 100 for inoculum stored at 5°C for 9 months. But those larvae exposed to the pathogen stored at 25°C for the same 9 months caused only 38% mortality for the first 24 hours, 42% mortality for the second 24 hours, bringing the mortality rate for 48 hours to 80%. A 14% mortality among the larvae during the third 24 hour period brought the total for 72 hours to 94% for this batch of inoculum.

The larvae exposed to inoculum stored at 5°C for 12 months suffered 59% mortality for the first 24 hours, 21% mortality for the 24-48 hours, resulting in a total mortality of 80% within 48 hours. An additional 11% from 48-72 hours resulted in a total of 91% mortality during the 72 hour period. The inoculum stored at 25°C for 12 months caused larval mortality of 64% within 24 hours, 21% from 24-48 hours, bringing the mortality level for 48 hours to 85%, with an additional 7% mortality between 48 and 72 hours. This batch of inoculum induced a total of 92% mortality during the 72 hours of the testing period. This

fluctuating pathogenic rate of Leptolegnia sp. stored at 5°C and 25°C for 3, 6, 9 and 12 months is shown in Figure 29.

An analysis of the combined effects of 5°C temperature and varying storage periods at each bioassay time indicated a declining pathogenic rate with a resulting mortality level of 87% within 3 months, 84% within 6 months, dropping to 69% at 9 months and further down to 59% at 12 months for a 24 hour assay time. For a 48 hour assay time, there was an initial increase in pathogenic rate producing mortality levels of 11% within 3 months, slightly increasing to 16% at 6 months and further increasing to 28 % at 9 months before a slight drop to 21% at 12 months. A 72 hour assay time showed a near 0 mortality level from 3-9 months and 11% mortality level at 12 months (Figure 30).

A similar analysis for 25°C showed a fluctuating pathogenic rate that induced 77% mortality level at 3 months, dropping to 63% at 6 months, and decreasing to 37% at 9 months and then rising to 64% at 12 months for the 24 hour assay time. For the 48 hour assay time the rate of pathogenicity, though low, steadily increased from 3-9 months and suddenly dropped from 9-12 months. The mortality level for this period was 18% at 3 months, 27% at 6 months, 42% at 9 months and dropped to 21% at 12 months. The pathogenicity for the 72 hour assay time produced 0%

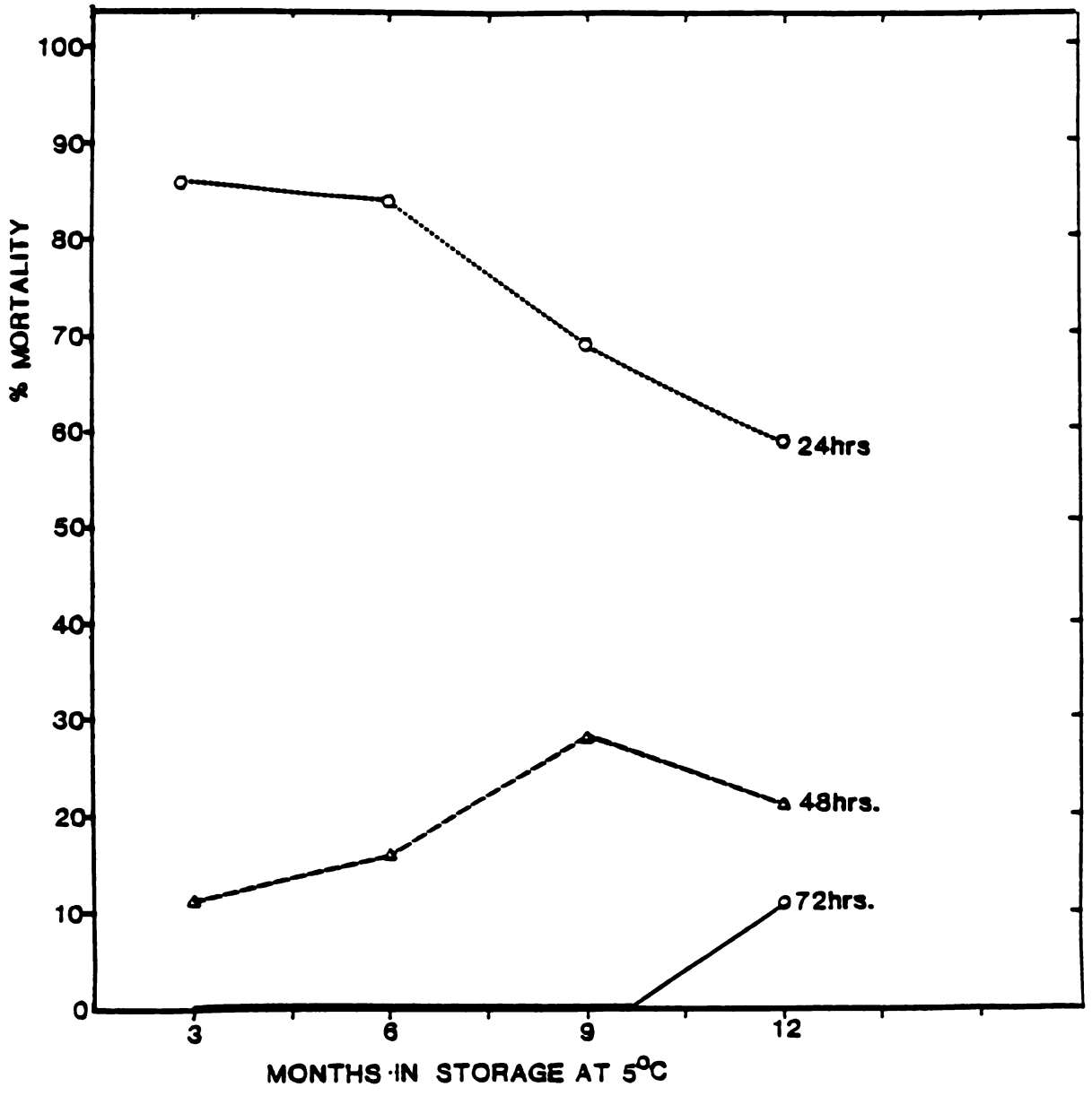


Figure 30. Percent mortality of Ae. triseriatus larvae infected with Leptolegnia sp. stored at 5°C for 3, 6, 9 and 12 months with larval mortality assayed at 24, 48 and 72 hours after exposure to pathogen (a density dependent model for 5°C).

larval mortality from 3-6 months, and a sudden rise to 14% at 9 months, and then dropped to 0% at 12 months (Figure 31).

A combined effect of temperature and storage period indicated that Leptolegnia sp. stored from 3 to 9 months at a temperature of 5°C, would maintain a high level of pathogenicity that would cause 98%-100% mortality level in two-day old larvae of Ae. triseriatus. While Leptolegnia sp. stored at 25°C for 3 to 9 months would maintain a level of pathogenicity that would produce 91%-98% mortality in the same larval age and species. Twelve months of storage at each temperature did not produce any significant difference in the overall pathogenic ability of Leptolegnia sp. established on split hempseed, but did indicate that a lower mortality level of about 91%-92% would be induced by Leptolegnia sp. stored at 25°C for 12 months (Figure 32).

Slant culture of Leptolegnia sp. maintained on Peptone Yeast Glucose (PYG) agar, supplemented with 10% glycerol, and PYG filled with mineral oil; Cornmeal Dextrose Peptone (CMDP) agar supplemented with 10% glycerol and (CMDP) filled with mineral oil sustained the viability of Leptolegnia sp. stored at temperatures of 5°C and 25°C for two years. Plain PYG, plain CMDP, Cornmeal (CM) agar whether plain, supplemented with 10% glycerol or filled with mineral oil did not sustain cultures of Leptolegnia

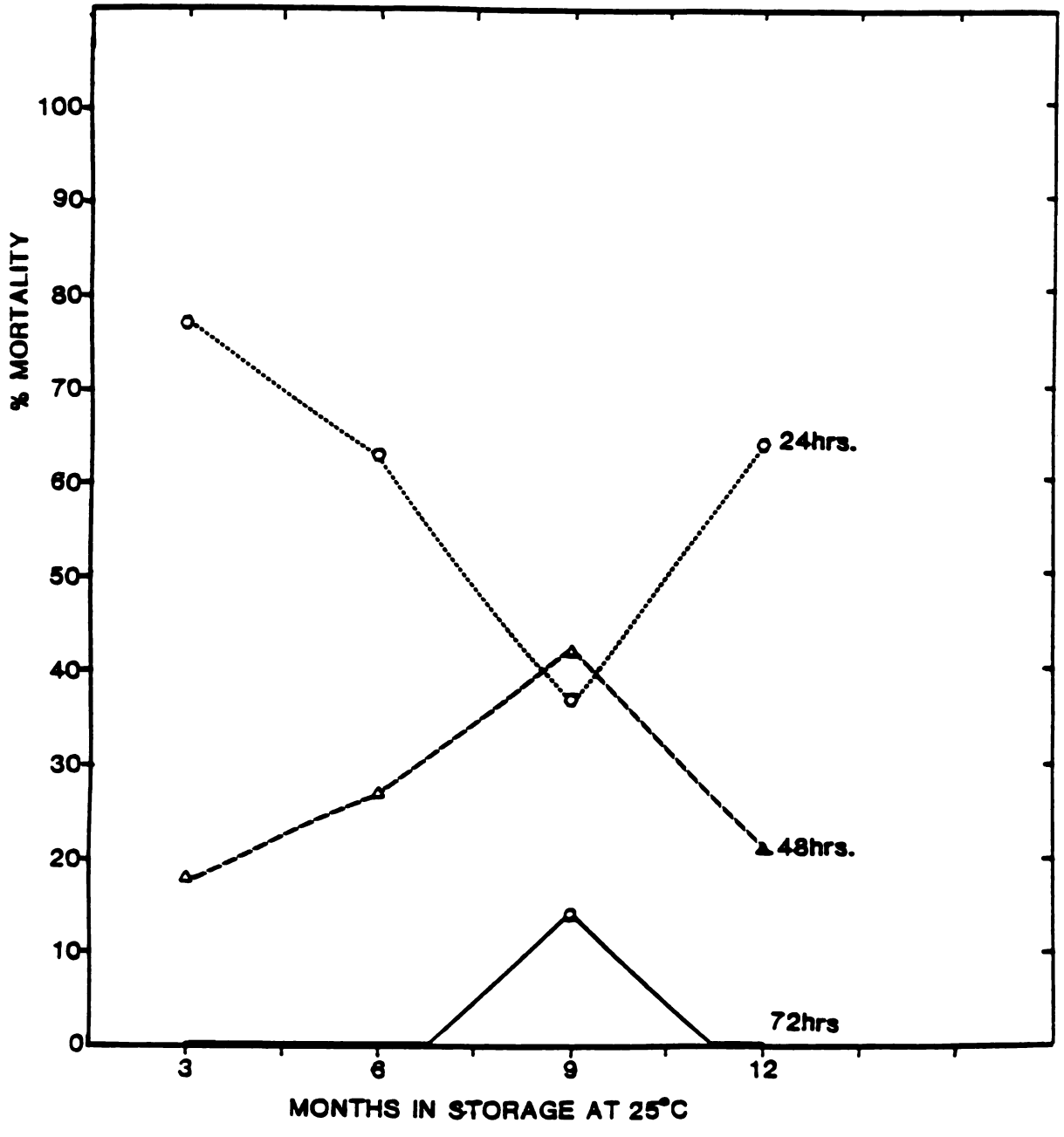


Figure 31. Percent mortality of Ae. triseriatus larvae infected with Leptolegnia sp. stored at 25°C for 3, 6, 9 and 12 months with larval mortality assayed at 24, 48 and 72 hours after exposure to pathogen (a density dependent model for 25°C).

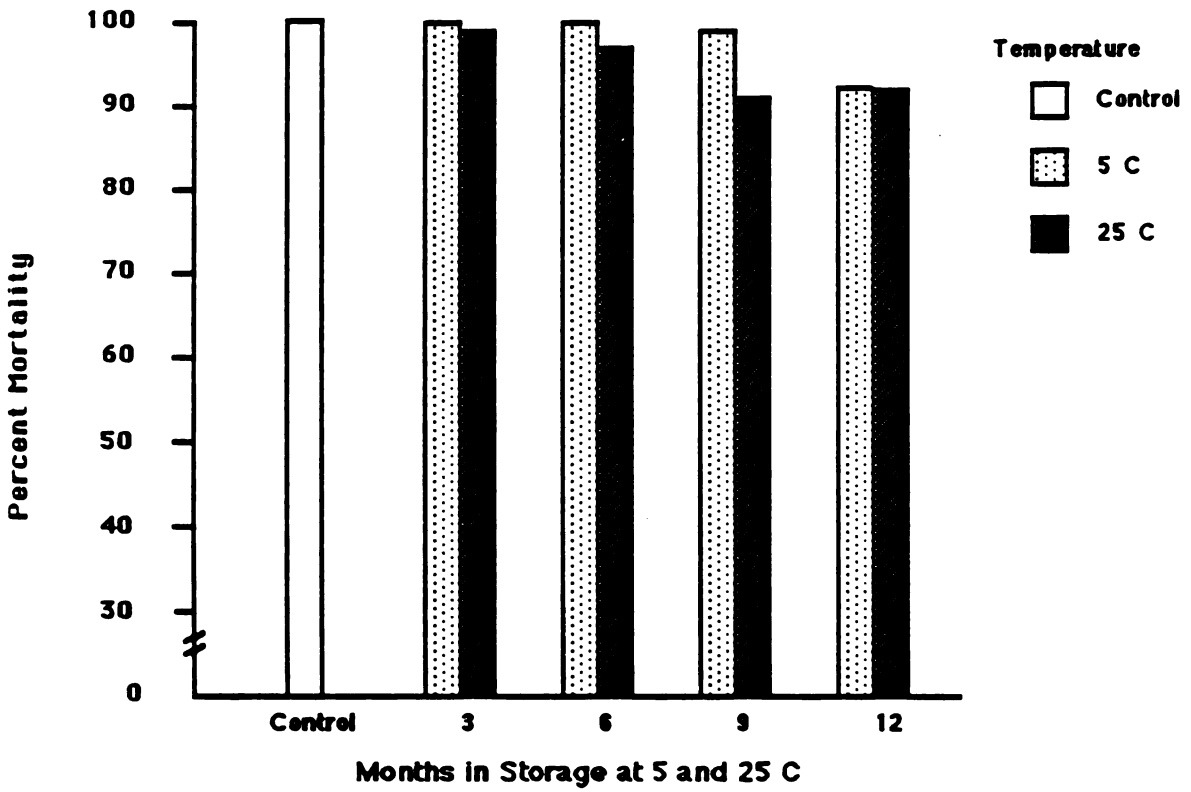


Figure 32. A comparison of percent mortality of two-day old larvae of Aedes triseriatus infected by zoospores of Leptolegnia sp. stored at 5°C and 25°C for 3, 6, 9 and 12 months (a measure of pathogenic potential).

sp. in a viable state under the same storage conditions (Table 7).

Table 7. Effect of storage temperature and growth medium on viability of Leptolegnia sp.

Storage temperature (°C)	Agar growth medium	Treatment and Results		
		Plain	Glycerol	Oil
5°	CM	-	-	-
	CMDP	-	+	+
	PYG	-	+	+
25°	CM	-	-	-
	CMDP	-	+	+
	PYG	-	+	+

Key

\+ = Viable
\- = Not viable
CM = Cornmeal
CMDP = Cornmeal-Dextrose-Peptone
PYG = Peptone-Yeast extract-Glucose

G. Pathology and Histopathology in Ae. triseriatus Larvae Infected by Zoospores of Leptolegnia sp.

Larvae of Ae. triseriatus attacked by zoospores of Leptolegnia sp. displayed abnormal behavioral patterns. Symptoms at the initial stage of infection was excessive grooming and wiggling. In uninfected larvae the anal papillae displayed distinct morphological patterns of string-like structures attached to circular structures

(Figure 33A). In infected larvae, the anal papillae were frequently extended and dilated, losing the characteristic patterns, while the mouthparts were moved frequently in a gaping fashion. The infected larvae gradually became sluggish, ceased feeding and eventually died, at which time the anal papillae either ruptured or shrank in size (Figure 33B). The larvae that were parasitized became darker in color and smaller in size compared to uninfected larvae. The infected, dead larvae settled to the bottom of the container within 12-24 hours of exposure, but subsequently floated on the surface of the water from 48-96 hours after exposure by which time masses of hyphae projected out of the bodies.

Gross pathology of the infected larvae included damage to the anal papillae, digestive tract, tracheae, fat bodies and muscles. Light micrographs of Glycol methacrylate (GMA) sections of an anal papillae of uninfected and infected larvae after 24 hours of exposure to the pathogen revealed gross distortion of the internal morphology (Figures 34A and 34B). The annular muscle (ring-like structures) suffered atrophy and disfiguration. The network of nerve and muscle fibers (fiber-like structures) that connect the annular muscles were destroyed for the most part, and the central canal system (lamella) within

Figure 33. Photomicrograph of external morphology of anal papillae of uninfected and infected Ae. triseriatus larva.

A. Uninfected anal papillae showing characteristic string-like and annular structures in each projection. Fifteen hours, control.

B. Infected anal papillae showing distortion and destruction of the annular and string-like structures and ruptured projections.

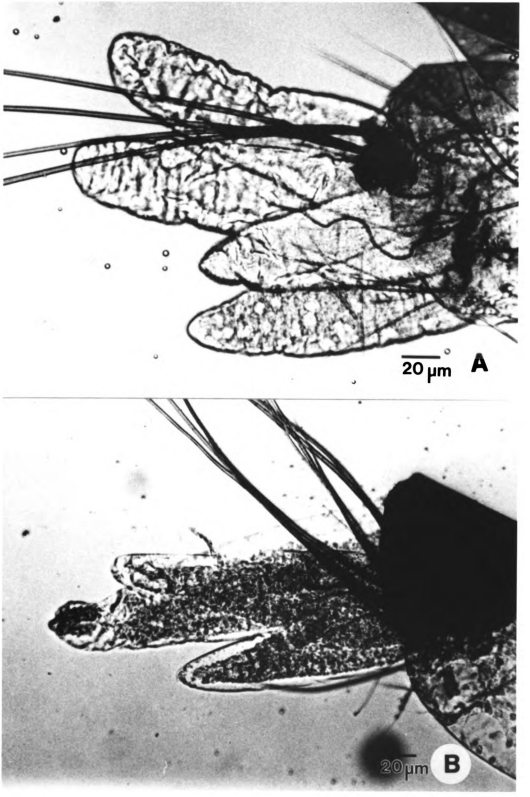
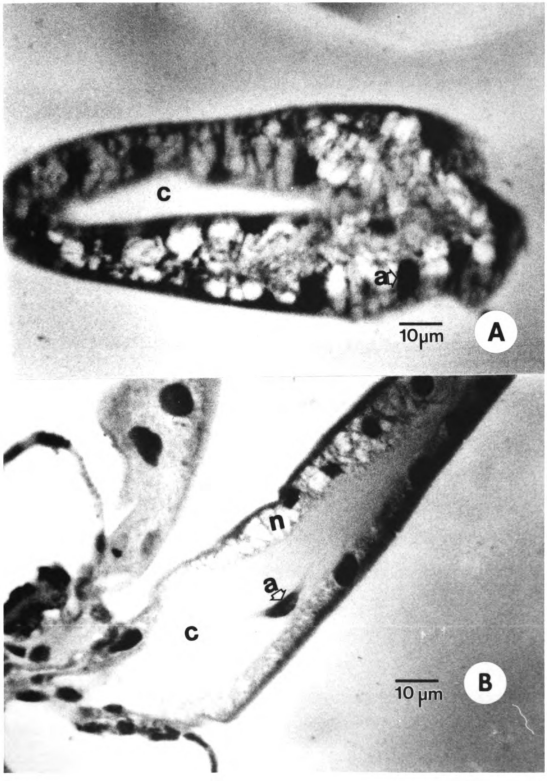

20 µm
A
20 µm
B

Figure 34. Photomicrograph of sagittal section of uninfected and infected Ae. triseriatus larva, showing internal morphology. Glycol methacrylate section, 5 micrometer thick.

A. Uninfected anal papilla showing characteristic annular muscle (a), central canal system (c) and network of muscle and nerve fibers (n).

B. Infected anal papilla 24 hours after exposure to the pathogen, Leptolegnia sp.

Note the disfiguration of the annular muscle (a), distortion of the network of muscle and nerve fiber (n) and enlargement of the central canal system (c).


c
a
10µm
A
n
a
c
10 µm
B

the anal papillae became enlarged, especially at its basal end.

The thoracic region of the infected larvae was generally engorged. A gross section of this region examined under a dissecting microscope revealed a large number of microscopic organisms. Three and five micrometer GMA sections of infected moribund and dead larvae 24 hours after exposure to the pathogen showed pathological damage to the foregut. In the dead larvae there was extensive vacuolation of the epithelial tissues in the foregut and midgut due to lysing of the cytoplasm and nuclei. Most of the epithelial tissues lining the esophagus and crop were completely destroyed (Figure 35A). A zoospore in a transitional motile stage was lodged in the basal membrane of the epithelial tissue (Figure 35A). There was accumulation of rod-shaped bacteria at the upper region of the crop where the esophagus terminates (Figure 35A). In the moribund larvae, there was accumulation of rod-shaped bacteria and zoospores in the crop (Figure 35B). A similar accumulation also occurred in the midgut, hindgut and rectum of both moribund and dead larvae, but accumulation and damage were greater in the dead larvae than they were in the moribund larvae. The teanidia of the trachea were also damaged in the infected larvae.

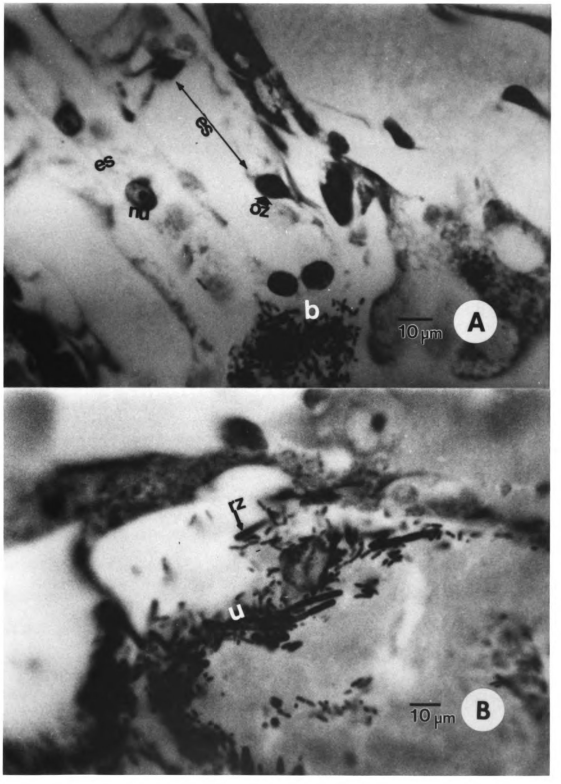
Figure 35. Photomicrograph of sagittal section of the larvae of Ae. triseriatus infected with Leptolegnia sp. Twenty-four hours after exposure to the pathogen, glycol methacrylate section.

A. Section through the esophagus of a dead larva 3 micrometers thick.

Note the zone of damaged epithelial lining (es) of the esophagus, distortion of the nucleus (nu) of epithetial tissue and accumulation of bacteria (b). An oval-shaped zoospore (oz) appeared to orient towards the muscle tissue.

B. Section through the crop of a moribund larva 5 micrometers thick.

Note rod-shaped zoospore (oz) and other zoospores (u) in close association with the bacteria.


es
ss
nu
dz
b
10 µm
A
z
u
10 µm
B

In uninfected larvae, layers of fat bodies bathed in the hemolymph between the integument and the gut. The fat bodies were surrounded by basal lamina-like structures. Uninfected larvae also have ganglionic structures strung together by ganglionic nerves lying below the integument. Twelve to twenty-four hours after exposure to the pathogen, the fat bodies were lysed, leaving gray spots in the hemocoel. A sagittal, 3 micrometer GMA section through the thoracic region, 24 hours after exposure (Figure 36A), showed a gray, lysed area of fat body, partially enclosed by dark laminal mass, a disfigured ganglionic body, a severed ganglionic nerve, and a cross section of a fungal hypha in the hemocoel adjacent to the destroyed ganglionic body (Figure 36A). The longitudinal muscle in this region was also damaged and the vacuolated epithelial lining of the stomodaeum was very evident in this micrograph (Figure 36A). A similar 5 micrometer section through upper abdominal region of moribund larvae showed damage to the glandular cells of the gastric caecum and similar glandular structures, expansion of the peritrophic membrane as its enclosed ventriculus became hyperplasic with an increased number of micro-organisms. A zoospore in a flagellated state was found lodged in the stomodaeal membrane, most of which were partially destroyed (Figure 36B).

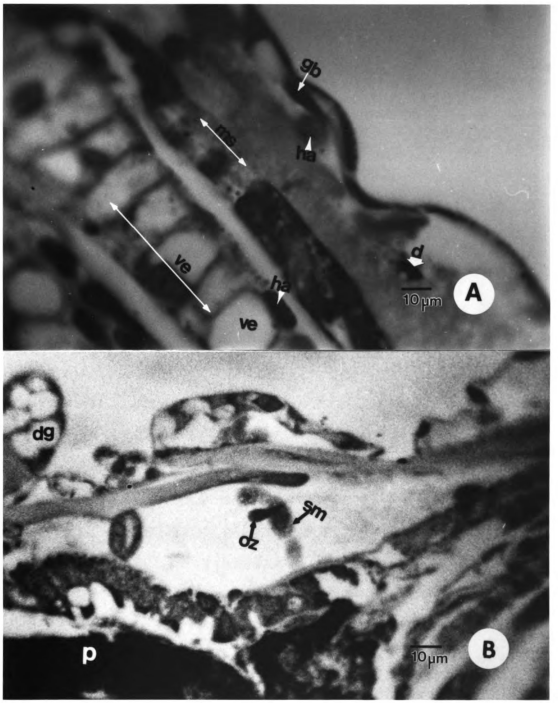
Figure 36. Photomicrograph of sagittal section of the larvae of Ae. triseriatus infected with Leptolegnia sp. 24 hours after exposure to the pathogen. Glycol methacrylate section.

A. Section through the thoracic region of a dead larva 3 micrometers thick.

Note vacuolation of columnar epithelial tissue (ve), a region of damage to the longitudinal muscle (ms), the destruction of fat body (d), disfiguration of the glandular structure (gb), and a section of hypha (ha) in the hemocoel.

B. Section through the abdominal region of moribund larva 5 micrometers thick.

Note oval-shaped zoospore (oz) lodged in damaged stomodaeal membrane (sm); disfiguration of ganglionic body (dg) in a portion of the gastric caecum, and enlargement of the peritrophic membrane (p).


gb
ha
ve
ha
ve
d
10 µm
A
dg
sm
dz
p
10 µm
B

An examination of a transmission electron micrograph (TEM) section of the head of infected larvae 12 hours after exposure to the pathogen, Leptolegnia sp., showed extensive damage to the mouthparts and muscles that guide movement of the mouthparts. A thick section through the head, showed that numerous zoospores had invaded the bundles of labial muscle (Figures 37A-C). Most of the muscle fibers were lysed by the invading zoospores creating a clear zone or lesion, while the uninvaded bundle remained intact and void of similar electron dense zoosporic structure (Figure 37B). An enlargement of the damaged area where the zoospore lodged showed many cellular structures were also destroyed and the zoospore seemed to be surrounded by what appeared to be a mucilagenous substance while inside the lesion (Figure 37C).

Further down in the head, a number of zoospores were lodged in the pharynx, most of them had invaded the epithelial tissue, and destroyed most of the cellular structures within that tissue (Figure 37D). A zoospore in its rod-shaped configuration was oriented toward the cuticulin layer that lines the pharyngeal cavity, separating it from the flat epithelial tissue that lines that tract (Figure 37D). Some encysted zoospores lodged within the lesion created through damage to the epithelial tissues and remnants of electron dense nuclear materials

Figure 37. Transmission electron micrograph of the head, thorax, and abdomen of Ae. triseriatus larva infected with zoospores of Leptolegnia sp. 12 hours after exposure to the pathogen, and a micrograph of uninfected larva as a control.

A. Section through the head, showing bundles of labial muscles in the cranial and the mouth (head) region of the larva, and showing invasion of some bundles by the zoospore.

B. An enlargement of two of the labial muscles to show initiation of liaison (la) damage by an invading zoospore (z).

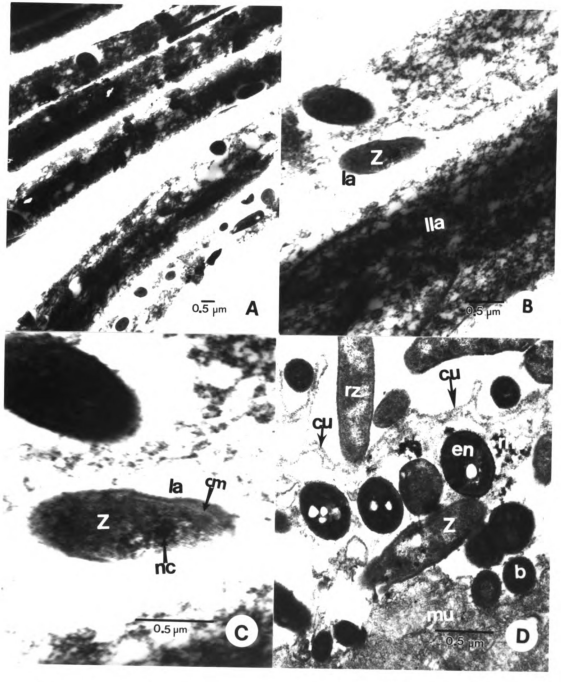
Note absence of such liaison (lla) in the uninvaded bundle.

C. An enlargement of invaded bundle to show extent of cellular damage (la) to the labial muscle and cellular structure of the zoospore.

Note the presence of nucleus (nc) and cell membrane (cm) in the invading zoospore (z).

D. Section through the head showing pharyngeal area.

Note the rod-shaped zoospore (rz) oriented towards the cuticle layer (cu) lining the pharyngeal tract. Also note an apparent portion of zoospore or germinated hyphogen (z) lodged against the muscle (mu) and the presence of bacteria (b). An encysted zoospore (en) rested in destroyed nucleus as denoted by remnants of electron dense nuclear material (rn).


Z
la
lla
0,5 µm
A
0,5 µm
B
la
cm
Z
nc
0,5 µm
C
rz
cu
cu
en
Z
b
mu
0,5 µm
D

could still be seen. Part of what appeared to be a growing hypha from a germinated zoospore lodged in the interstitial layer between the muscle and the epithelial layer which had been destroyed (Figure 37D). Some bacteria were associated with the zoospores (Figure 37D).

Sections through the thoracic and abdominal regions also show damage done to the epithelial layers, and the muscle (Figures 37E-H). A structure that matches the size and description of a zoospore in its oval configuration lodged in the longitudinal muscle of the thorax, ripping the muscle from its base and creating a lesion (Figure 37E). A large part of the muscle fibers has been lysed, leaving clear zones upon which the zoospores encysted (Figure 37F). In the uninfected larvae, the muscle fibers appear as a continuous sheet with interconnecting penultimate bands of myofibrils, and very few intercellular spaces between them (Figure 37G). In the infected larvae, 12 hours after exposure to the pathogen, a section through the abdomen showed extensive damage to the myofibrils so that the muscle layer became highly vacuolated (Figure 37H). The electron dense nuclear materials in the muscle and other cellular structures were lysed.

It was observed that mosquito larvae exhibited a self defense mechanism to infection caused by the zoospores of Leptolegnia sp.. Dissection with dissecting microscope

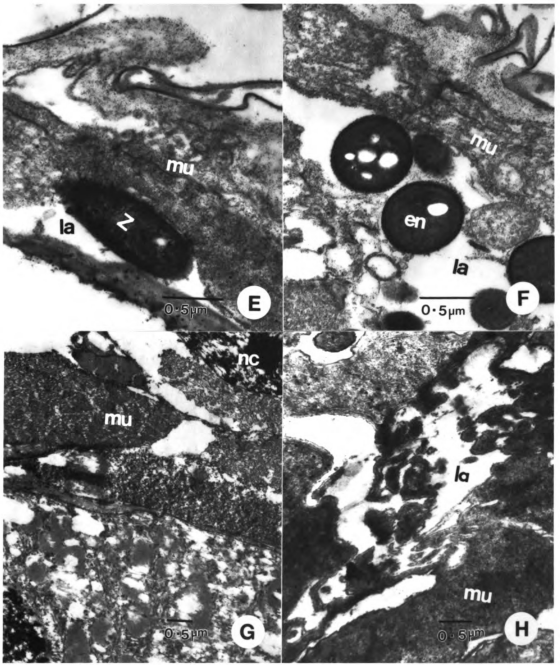
Figure 37. E. Section through thorax showing zoospore (z) invading the muscle (mu) layer of the larval host, and leaving a zone of lesion (la).

F. Section through the thorax showing encysted zoospores (en) in damaged areas of the muscle (mu).

G. Muscle tissue of an uninfected larva showing the continuity of the sheets of muscle (mu) and the connection by electron dense myofibrils, leaving very little intercellular spaces.

Note the presence of nucleus (nc) and electron dense nuclear materials.

H. Muscle layer of an infected larva showing excessive destruction of the muscle (mu) layers leaving large spaces (la) between layers and pockets of muscles. The myofibrils were destroyed.


mu
la
Z
E
0·5μm
mu
en
la
0·5μm
F
nc
mu
G
0·5μm
la
mu
0·5μm
H

showed possible melanization within the hemocoel, as evidenced by darkening of the encysted zoospores and sporangia within the hemocoel (Figures 21A and 21B).

DISCUSSION

Unlike its counterpart, Lagenidium giganteum, Leptolegnia sp. has received very little attention as a mosquito pathogen. Yet its virulence in parasitizing mosquito larvae seemed to be comparable to that of Lagenidium giganteum, another aquatic Oomycete fungus, facultatively parasitic on mosquito larvae. As observed in this study, Leptolegnia sp. demonstrated a very high pathogenic potential and pathogenic rate against two-day old larvae of Aedes triseriatus. As observed in all experiments, this pathogen induced a 100% mortality within 48 to 72 hours in all larval populations tested. This observation agrees with the report of McCray et al. (1973) that Lagenidium culicidum killed all the larvae of Aedes and Culex species tested within 48-72 hours.

In all tests conducted, the highest mortality occurred within 24 hours, indicating a high rate of infectivity within this period. Such a high rate of parasitization may be a direct response to high host density and also high density of zoospores released. Coker (1923) noted the pathogenic potential of Leptolegnia sp., but its ability to reduce host population was not recognized till Vallin

(1951) reported induction of epizootics in copepods by Leptolegnia sp. It was observed in my studies that infection did not only occur from the unit of inoculum, but also from the infected larvae to healthy ones. Although the cadavers were not used as a unit of inoculum in our tests, it is envisaged that such an occurrence could induce epizootics in a natural population, enhance persistent infection under field conditions and also reduce the need of re-application, a factor that will in turn result in low cost to the pest control program. McCray et al. (1973) reported 100% mortality in healthy larval populations of Aedes and Culex species inoculated with cadavers of larvae killed by Lagenidium culicidum renamed Lagenidium giganteum (Umphlett 1973). Observations made from these studies indicate that Leptolegnia sp. has a high rate of virulence, infectivity, survival and dispersal; properties which Tanada (1963) cited as being significant in epizootiology, but further studies are needed to elucidate these characteristics.

In the bioassay test, 50% of the population of Ae. triseriatus larvae tested were killed within eleven hours, illustrating a high susceptibility of Ae. triseriatus larvae to infection by Leptolegnia sp., a pathogen already noted for inducing 80% mortality in nine other species of mosquito larvae in addition to Ae. triseriatus (McInnis et

al. 1985). Induction of 96% mortality within 24 hours and 100% mortality within 48 hours, as observed in these studies, supports the findings of McInnis and Zattau (1982), who reported 100% mortality among larvae of Aedes aegypti infected by Leptolegnia sp.. Most fungal pathogens of mosquitoes have been reported to accomplish infection within 24 to 72 hours. Couch and Romney (1973), Umphlett and Huang (1972) and Washino (1981) found that Lagenidium giganteum accomplished infection within 24 to 48 hours after exposure to mosquito larvae. Sweeney et al. (1983b) reported that larvae of Culex annulirostris succumbed to infection by Culicinomyces clavisporus within 24 to 48 hours of exposure.

The bioassay study has further demonstrated that the potential of Leptolegnia sp. to decimate populations of mosquito larvae was not hindered by the use of established hyphae and sporangia on the half split hempseed as an inoculation unit. That the split hempseed inoculation unit induced 100% mortality among Ae. triseriatus larvae illustrated increased production of zoospores as reported by Seymour (1978). This study is similar to the findings of McCray et al. (1973), that the fungus, Lagenidium culicidum established on hemp seed could infect mosquito larvae. In previously reported studies, infections of mosquito larvae with Leptolegnia sp. were accomplished

either by agar culture disks (McInnis and Zattau 1982) or by use of mycelia collected from agar broth (McInnis et al. 1985). The use of this split hemp seed inoculation unit establishes another technique by which the pathogen could be effectively applied. And its success under field conditions needs to be fully investigated.

In nature, Leptolegnia overwinters in insect exuviae, seeds, twigs of plants and other organic debris in the substratum (Sparrow 1960). Therefore, the use of split hempseed technique may be advantageous in simulating nature's way of preserving the propagule and consequently the inoculum for continued infectivity. It is also a very inexpensive and simple technique that may be applicable both in residential areas and in small-scale operations. As noted by other researchers (McInnis et al. 1985, WHO 1985), Leptolegnia sp. exhibits declining pathogenicity when cultured on artificial media for a long period of time. This problem was somehow overcome by occasional transfer to alternate growth media. But the use of the split hempseed technique may offer a solution to that problem.

Since hempseed is not commonly available, the use of similar dicotyledonous seeds needs to be explored. In a very small trial, I found that Leptolegnia sp. became established on sunflower seeds but in these studies the

established sunflower seed culture was not used for infection. Perhaps this needs to be explored. The disadvantage in this technique is that the lethal dose cannot be established, since it is difficult or almost impossible to quantify or predetermine, prior to application, the total number of zoospores that may be released from each inoculation unit. But this is a common problem with any of the other two techniques used in applying Leptolegnia sp. (McInnis et al. 1985). Nevertheless, a rough estimate can be made by using a hemocytometer to count zoospores in one cubic centimeter from similar inoculation unit prior to application.

In laboratory studies, Leptolegnia sp. has the ability to persist and remain pathogenic to five sequential batches of Ae. triseriatus larvae. This characteristic makes it an enticing potential candidate for the control of Ae. triseriatus and other multivoltine mosquitoes. Similar persistence under laboratory conditions was reported by Frances et al. (1984) for Culicinomyces against two species of mosquitoes. Field experimentation is necessary to determine if either these Leptolegnia or the Culicinomyces would be control agents with the persistence reported for Lagenidium giganteum by Washino (1981), McCray et al. (1973), Christensen et al. (1977), Glenn and Chapman (1978), Jaronski and Axtell (1983).

The results of experiments on persistence and bioassay have produced baseline information that the split hempseed technique might be an effective method of infecting natural populations of mosquito larvae. With this technique the pathogen is capable of persisting in the environment and releasing zoospores for a period of at least 27 days. Since Leptolegnia is a eucarpic fungus, the use of this type of inoculation unit, with mycelia at different maturity levels, would enhance a gradual release of zoospores directly into the environment for an extended period of time and would be expected to reduce the mosquito populations to a level that minimizes their vector potential.

The orientation study illustrates that zoospores of Leptolegnia sp. orient themselves in relation to mosquito larvae. The fact that encystment occurred on all three treatments used in this study confirms previous findings that zoospores of Leptolegnia sp. escape from the sporangia, swim, encyst and adhere to a surface of the host or to the substratum (Petersen 1910, Couch 1924, McInnis 1982). This observation was also made throughout the course of this investigation. An analysis of the encystment data reported in Table 4 showed that only 7% of the zoospores encysted on empty tubes while 93% encysted on tubes that contained media (mosquito homogenate + plain agar) and 77%

of those 93% zoospores that orientated to media , encysted on tubes that contained homogenate media while 16% of them encysted on tubes that contained plain agar media.

When analyzing the total encystment 72% of the zoospores encysted on homogenate while 21% encysted on plain agar, this represents (by substraction) a 51% increase in encystment on larval homogenate over the plain agar. The phenomenon of higher encystment on tubes that contain media over tubes that were void of media indicates positive orientation to some organic substrate upon which encystment must occur. The fact that the highest encystment occurred on tubes that contained larval homogenate than on tubes that contained plain agar media indicates that the larval homogenate elicited some stimulus to which the swimming zoospores responded and orientated. Swimming, adherence and encystment are well-known phenomena in zoospores of other Oomycetes fungi (Zentmyer 1970, Hickman 1970, Young et al. 1979, Carlile 1975). Such behavior has not been reported for fungal pathogens of mosquitoes.

Data in this experiment show a strong evidence of orientational response of zoospores to a stimulus in the homogenate media. Since the particular stimulating substance is not known and the zoospores were not watched in action to determine their pattern of movements, further studies are needed to determine details of the behavioral

pattern exhibited in this study. Behavioral differences associated with the larval homogenate supports the findings that zoospores orientate themselves in response to chemical stimuli in their environment (Cameron and Carlile 1978, Khew and Zentmyer 1973, Hickman 1970, Nolan 1983). More encystment occurred on the surface of the media than on the glass tube. This supports the experimental hypothesis that swimming zoospores orientate themselves in response to stimuli elicitated from the surface on which they encyst and adhere.

The behavior of zoospores in this orientation study offer an incentive for investigations on the host searching ability and host finding potential and efficiency of zoospores of Leptolegnia sp., especially as it is being evaluated as a potential mosquito larvicide (WHO 1977). A study to elicit the specific substance to which the zoospores respond and the receptor organ(s) involved would be beneficial. It is possible that like plant pathogenic fungi, insect pathogenic fungi respond positively to stimuli that would enhance their nutritional, developmental and dispersal needs. More studies could elicit such information and might be beneficial in formulating this pathogen for use as a biocontrol agent.

Although not indicated in Table 4, a comparison of the data collected from the ends and middle of the tubes in

each treatment indicated that zoospores encysted more in the center of the tubes than on the two ends combined. The homogenate medium had 61% encystment in the center compared to 39% on the end, the plain agar medium had 55% in the center compared to 44% on the end, and the tube that was void of solid medium (empty) had 89% encystment in the center compared to 11% encystment on the end, a significant difference in each treatment. Since higher encystment in the center was uniform in all three treatments, it was probably not an orientational respond to any factor from the medium on which encystment occurred but rather, a respond to some form of an environmental factor such as light reflection or some disturbance generated in the water medium. Further studies are needed to explain the above observed phenomenon which might shed some light into the behavior of the zoospores of Leptolegnia sp. and the factors that affect such behavior.

Zoospores are motile, and knowledge of their behavior would be helpful not only in formulation but also in determination of time and mode of application. If light reflections or disturbance could cause zoospores to retreat and encyst in a relatively shaded or protected area, then that characteristic would be beneficial in tree hole situations. It would be anticipated that encystment would be higher in organic detritus at the bottom of the tree

hole. Such placement would keep encysted spores in close proximity to newly hatched larvae for filter feeding in the tree hole.

Observations made in the study on mode of infection were contrary to the generally held notion that most pathogenic fungi infect their insect hosts by producing spores which penetrate insect cuticle by means of a penetrant tube. Once inside, the penetrant tube or invasive hypha then ramifies through the insect hemocoel, (Poinar and Thomas 1984, DeBach 1973, Steinhaus 1949, 1963, Weiser 1969, McInnis and Zattau 1982 and Sweeney 1975). This generalization may be common among the higher fungi whose spores (conidia) are capable of adhering to the surface of a host upon which attachment can occur. No adherence mechanism has so far been reported in either higher or lower fungal pathogens of mosquitoes. McInnis and Zattau (1982) identified two modes of infection, cuticular encystment by secondary zoospores, and germination of zoospore cysts in the alimentary canal. They did not state the time sequence of these two infection mechanisms. They also stated that cuticular invasion occurs in all larval ages, with encysted spores attached to the thorax, abdomen and anal papillae, but it was not clear whether the observed spores were attached before or after the larvae were dead.

It was observed in this study that the spores of Leptolegnia sp. did not remain on the larval integument long enough to either produce a penetrant tube to mechanically push through intact integument or secrete an enzyme that might lyse the integument of healthy larvae prior to invasion by a germinating hypha. The spores that encysted on the integument showed a tendency to fall off with the slightest disturbance to the water medium. The rate at which newly infected larvae groom themselves, spores without any attachment mechanism would be wiped off. Self grooming is a natural behavior in mosquito larvae, but it was observed that the larvae that had spores on the integument or were feeding on encysted spores, tended to groom more rapidly and frequently. During this process the spores on the integument would fall to the bottom of the dish while the spores in the bucal cavity would still be lodged there, or be rolled down the digestive tract by the peristaltic action of the pharynx.

It would appear that if the spores on the integument had any adherence mechanism, or were in the process of invading the larval body, they would not fall off so easily. Grooming among these challenged larvae appeared to be a self defense mechanism. Increased palpitation and gaping movements of the mouthparts by these groups of larvae were indicative of possible suffering from spores

within the bucal area, rather than from spores wiped off from integument.

The mode of infection of Leptolegnia sp. observed in this study on the larvae of Ae. triseriatus was primarily through ingestion of encysted spores and mycelial or sporangial fragments. McInnis and Zattau (1982) observed ingestion of zoospore cysts and reported the subsequent germination of spores from the midgut as being the common mode of infection in early larval instars. McCray et al. (1973) reported that motile zoospores of Lagenidium culicidum were ingested by the larval host. Petersen (1910), Carpenter (1982) and Sweeney (1983) also reported ingestion of spores as being the portal route of infection by other fungal pathogens of mosquito larvae. Burges (1981) remarked that aquatic insects become susceptible to infection through filter feeding and ingesting inert stages of pathogens and particulate toxins. McInnis and Zattau (1982) reported the presence of spores in anal canal, thorax, abdomen, head, anal papillae and the production of invasive tube, but they did not specify at what time in the pathogenic process those various observations were made. In my studies, spores were also seen in the anal canal, but it is not known how they got there.

It was also observed that after the larvae had been weakened or killed through internal infection by ingested

spores that the invasive tube from the integument and numerous encysted spores on the thorax, abdomen, head and anal papillae were subsequently observed. Also in my studies, cystic ingestion, moribund behavior and death occurred within 12-24 hours, cuticular encystment, occurred between 24-48 hours and a few penetrant tube invasions, more encystments, and hyphal projections from the body occurred at 48-72 hours. The relationship of the invasive tubes at this point appears to be saprophytic rather than pathogenic, but this needs to be determined by further research.

The two-day old larvae fed heavily on the inoculation unit and developed mycosis shortly thereafter. Examination of the integument at that early stage failed to show any invasive tubes or wounds, but the internal organs, including the hemocoel, at that time showed different developmental stages of the fungus in various organs, with the largest accumulation in the digestive tract. In moribund larvae, cessation of feeding, the distortion in the morphology of the gut epithelium, and the erosion of the gut microtubules or brush border all seem to indicate damage to the digestive tract either by the spore or zoospores or by the associated bacteria. An experiment involving microfeeding of only the spores directly to the larvae might help to underpin ingestion as the principle

mode of infection. Microfeeding might be a very difficult experiment because the zoospores are fragile and the encysted zoospores become motile in a very short time. McInnis and Zattau (1982), noticing hyphal development in the hemocoel, referred to the disease induced by this fungus on the mosquito larvae as coelomomycosis. Findings in this study indicate that disease actually starts from the digestive tract and spreads to the hemocoel where numerous zoospores in motile or encysted stages were commonly found either attacking the fat bodies, being attacked by the hemocytes, or germinating into hyphae.

Zoospores were found moving in the hemocoel but the mechanics via which they got to the hemocoel, though not fully understood, does not appear to be through penetration of the integument because, at that period (6-12 hours after inoculation) there was no wound on the integument and the larvae were moribund but showing symptoms of infection (palpitation, sluggishness and others as described in the result section). It could be speculated that when an ingested primary zoospore gets to the midgut, it rests for a while then returns to the secondary zoospore and exits through the epithelial lining of the gut to get to the hemocoel where it swims for a while before encysting. The presence of primary encysted spores in the hemocoel might be due to ingestion of motile zoospores which pass through

the lining of the gut epithelium and encyst in the hemocoel. McCray et al. (1973) reported that ingested motile zoospores of Lagenidium culicidum could enter tissues of the digestive tract of a larval host. Figures 36B and 37B in this study confirm similar observation for zoospores of Leptolegnia sp.

There have been no previous reports about the presence of encysted spores in the trachea, but as illustrated in Figure 13 numerous encysted spores were seen in the trachea fifteen hours after infection. It is not clear how the spores got in there especially since the trachea is not known to contain any water or fluid, except for a very brief period during ecdysis (Chapman 1982). However, pneumatisation follows so rapidly that it is not known if sufficient time could have been allowed for the zoospore to swim into the trachea during ecdysis. It could be speculated that some other factor(s) might have caused physiological stress on the larvae resulting in loss of control of the spiracular valve. Consequently, water could enter into the trachea through the spiracle, and this might facilitate entrance of zoospores into the trachea. This speculation is based on the observation that in moribund larvae the trachea becomes darker in color, wiggling movement is highly reduced and a horizontal rather than vertical position is assumed in the water.

The more likely portal route into the trachea is from spores already in the hemocoel, attaching to the tracheal wall and penetrating through there. This is evidenced by the presence of rod and oval shaped (motile) zoospores in the trachea, and dark wound spots (Figure 18) on the wall of the trachea, denoting, as McCray et al. (1973) and McInnis and Zattau (1982) stated, site of invasion of zoospore. Observation of various stages in the life cycle of Leptolegnia sp. (Figure 5) at the initial stage of the study, was very critical in determining the invasive phases of this pathogen in the larval host. These observed life cycle stages in Leptolegnia sp. is similar to those reported by Petersen (1910), Coker (1923) and Seymour (1984) for named species of Leptolegnia.

Biological control of mosquitoes with fungi is so much in its infancy that the actual mode of action of the fungus has not been investigated. In addition, there are so very few researchers involved in culicid mycosis that many intricacies are yet to be resolved. A number of observations, which warrant future investigations, were made in the course of this study. Every sample of infected mosquito larvae examined had numerous rapidly reproducing rod shaped bacteria (Figures 18 and 35) in close association with the fungal pathogen. Although the actual role of these bacteria and their associations with this fungus

are yet to be discerned, it could be speculated that they are symbionts. The gross morphological and histological changes in many infected larvae and the extrusion of the peritrophic membrane in some infected larvae, especially those whose midgut became highly extended with a large accumulation of bacteria and fungal spores, are suggestive of the presence of some toxic substances or enzymes in the system. The presence, nature and source of such toxicants need to be investigated. Such substance could create a physiological stress on the larvae, thereby facilitating entry of spores through openings other than the mouth, or penetration of an invasive hyphae through the integument.

The realization that ingestion is the primary mode of infection in this filter-feeding aedian species stimulated an investigation on the effect of starvation on larval susceptibility. The compelling reason for this experiment was the conceived idea that since the infective units (zoospores) are ingested, susceptibility level could be reduced with increased availability of other food sources, and increased with food scarcity. The results clearly contradicted this hypothesis, in that there was no significant difference in the mortality rate or level between starved and well fed larvae. The starved larvae were given two separate exposure times with the notion that the longer exposure might increase total ingestion,

resulting in an increased potential for infection. Again, the experimental results were opposite to the expected results, as exposure time had no effect on susceptibility. The full implications of the impact of food availability and scarcity cannot be assessed from this one experiment. More experiments with changes in methodology and the use of carefully selected parameters need to be conducted. Perhaps quantifying the innoculum or using a serial dilution technique with the inoculum would produce the expected results, or confirm the present findings.

Storage is one of the problems that must be resolved before any fungal pathogen of mosquito can be formulated or selected for field application, (Heimpel 1972, McCray et al. 1973, WHO 1979). The storage phase of my investigation was undertaken to provide some data that might contribute to the solution of the problem. The ensuing discussion analyzes the results on the viability of the stored inoculum, effect of temperature and length of time in storage on the pathogenic potential and pathogenic rate of the stored inoculum, when used for infecting two-day old larvae of Ae. triseriatus. Larval mortality was used as an indirect index for measuring pathogenic potential and rate following infection.There is no reported literature on storage of Leptolegnia or any other fungal pathogen of

mosquitoes. The data is interpreted based on experimental hypothesis and the biology of the pathogen.

The results obtained in investigating the effect that storing the split hemp seed inoculation unit at 5°C and 25°C for 12 months might have on the pathogenic potential of Leptolegnia sp. indicated that the potential of this pathogen to parasitize mosquito larvae within 72 hours after being stored under the conditions described above was not greatly reduced. It showed that in a 72 hour post exposure assay, inoculum stored for 12 months at 5°C, produced a sufficient number of zoospores to infect and cause 91% mortality among two-day old Ae. triseriatus larvae, while the units stored for 12 months at 25°C produced less zoospores, and induced 90% mortality in a similar host population within 72 hours post exposure. Such pathogenic potential did not represent a statistically significant reduction when compared with 100% mortality induced by the freshly prepared inoculation unit used as a control (Figure 32).

The pathogenic rate of zoospores released from units stored at 5°C from 3 to 9 months was faster,killing 59-86% of the larvae within 24 hours, while those stored at 25°C killed 38-77%. In comparison with the control, the rate of pathogenicity of the inoculum stored at both temperatures was slower. The control killed all the larvae within 48

hours, while the stored inoculum, in general, required 72 hours to kill all the larvae (Figure 29). The slow rate of pathogenicity among the stored inoculum is probably because some hyphae died during storage, resulting in a low density and slower development of zoospores. The mortality level obtained from inoculum stored at 25°C was better than was expected because microscopic examination of the hyphae on the split hemp seed prior to inoculating showed more hyphal death than in the inoculum stored at 5°C. Viability, pathogenic potential and rate obtained from inoculum stored at 5°C was as expected because microscopic examination and agar culture test showed several hyphae on the split hemp seed were still alive at the end of the 12 months. Besides, several isolates of aquatic fungi have been maintained on split hemp seed in water at temperature of 5°C for many years by replacing the water and adding new hemp seed yearly (Beneke, personal communication and observation).

The study on the effect of storing the inoculation units for 3, 6, 9 and 12 months at 5°C and 25°C indicated that, in general, the pathogenic potential of stored inoculum gradually decreased in response to an increase in the number of months the inoculum was stored, but this gradual decrease in percent was more obvious in the inoculum stored at 25°C in which low potential caused a

reduction in mortality level from 99% during 3 months of storage to 90% during 9 and 12 months of storage (Figure 32). The decrease in pathogenic potential was due to a reduction in the numbers of zoospores produced, and this reduction was probably due to the observed death of many hyphae and formation of gemmae caused by a long period of storage in the same medium.

The pathogenic rate of the inoculum also gradually decreased for the first 24 hours after exposure, with the increase in months of storage, especially at the 9^{th} month when a low rate (38%) was followed by an increased rate (64%) at the 12^{th} month. The mortality rate for the 48^{th} hour increased greatly at nine months (Figure 29). The possible explanation for this is that while some hyphae were dead, many of them had formed gemmae. The few viable hyphae or gemmae in the culture formed sporangia whose zoospores killed the larvae 24 hours after inoculation. The gemmae that were formed, later developed zoospores or hyphae from which sporangia were formed to release zoospores which killed more larvae during the 24^{th} to 48^{th} hour. The possible role of gemmae in increasing zoospore density, with an increase in assay time is illustrated in Figure 29, where a low pathogenicity of storage at $25^{o}C$ for 9 months during 24 hours, was markedly increased during 48 to 72 hours.

The mortality pattern produced after 9 months storage is different from the general pattern found throughout the course of this study. Generally, a greater number of larvae died during the initial 24 hours of exposure than died during the second 24 hours, bringing a high total number of larvae dead within the cumulative 48 hours. Then a smaller number of larvae died at the third 24 hour period increasing the number of dead larvae during the total 72 hour duration of the experiment. A possible explanation for this usual mortality pattern is that a large number of zoospores were released during the initial 24 hours of exposure when there is a higher number of larvae, so more larvae were killed. This observation is similar to the statement of Maddox (1984), that increase in pathogenic organisms result in increase in infection and mortality.

During the 24 to 48 hour exposure, fewer larvae remained for the fewer zoospores, consequently, fewer larvae are killed. Then during 48 to 72 hour of exposure, even fewer larvae are still alive for infection by the zoospores so very few larvae were killed. In cases where all the larvae are killed during the 24 to 48 hours exposure periods, no larva was left to be infected by the zoospores still present. This sequence followed the normal expectation in the population dynamics of host parasite interaction, that when the host density is high the rate of

parasitization is high, and when the host density is low, the rate of parasitization is low. So under normal circumstance the pathogenic pattern of Leptolegnia sp. is density dependent. Moore-Landecker (1982) reported that parasitic fungi are most effective as biological control agents under high density of hosts and parasites.

This being the case, the most plausible explanation for the occurrence at the 9 month period is that the batch of zoospores that infect the larvae during the second 24 hours is not from the same population as the batch that infected during the first 24 hours. It is assumed that zoospores released from germinated gemmae or new hyphae on the hemp seed are responsible for a large proportion of the mortality seen at the 48 and 72 hour periods after 9 and 12 months of storage. Future research should seek to collect gemmae from stored units and use them as inoculum. If indeed gemmae are capable of zoospore formation, as speculated or suggested here and of inducing meaningful mortality level in the host, then it might be more feasible to mass produce, store, transport and dispense large quantities of inoculum in a less fragile, but still infective stage. Formation of gemmae and possibly oospores may be responsible for the lack of significant difference in the pathogenic potential between freshly prepared

inoculation units and inoculation units stored at various temperatures for various lengths of time (Figure 32).

Leptolegnia sp. established on split hempseed produces hyphae at different maturity levels. Some of the hyphae may form sporangia while others are just beginning to grow. Storage of such a unit would be advantageous in that it guarantees the availability of a viable inoculation unit at some stage of development even though some portions of them die while in storage. The presence of organic matter (the hempseed) serves not only as a substrate but also as a medium for overwintering under hazardous environmental conditions. Under unfavorable conditions, this pathogen forms gemmae and possibly thick wall, resistant oospores. The storage technique used here would enhance the formation of any or all of these structures should the growth medium become depleted. Results from this study on storage may have serious implications on the field application of this pathogen. A careful correlation of the biology of the mosquito host with that of the pathogen, coupled with meaningful application of information obtained from this study, may enhance a decision on the form and time of release of pathogen, and also on the prediction of the mortality level to be attained from stored inoculum.

The study on the effect of growth media on viability of stored Leptolegnia sp. indicated that CMDP and PYG would

support slant cultures of the pathogen for up to two years. Nolan (1983) has found that a species of Leptolegnia utilizes glucose for a carbon source and also require organic nitrogen for its nutritional requirements. The result of my study indicates that a nitrogen source may be required by Leptolegnia sp. in storage. The peptone and dextrose in cornmeal (CMDP) agar and peptone, yeast and glucose agar (PYG), respectively, must have provided Leptolegnia sp. with the needed carbon and nitrogen sources. In storage, the 10% glycerol in the media reduced protoplasmic collapse and the mineral oil prevented desiccation in the agar slants. A combination of these factors enhanced the survival of Leptolegnia sp. stored at temperatures of 5°C and 25°C for up to two years without losing viability and pathogenicity.

The ability of inoculation units of Leptolegnia sp. to remain viable and pathogenic after 12 months on hemp seeds in water that was not replenished and the slants on CMDP and PYG after 24 months in storage at 5°C and 25°C qualifies Leptolegnia sp. as an effective insect pathogen in accordance with the requirements set by Heimpel (1972) and McInnis and Zattau (1982). Like Achlya (Johnson 1956), Leptolegnia sp. can also be established on split hemp seed and stored at room temperature but, unlike Achyla, it does not need a new culture with fresh split hemp seed every 6

or 8 months. Leptolegnia sp. can be maintained for 12 months with the same hemp seed and in the same sterile distilled water. Though both the split hemp seed technique and the slant culture method have proven effective in storing Leptolegnia sp. in a viable and pathogenic stage, mass producing, storing, transporting and dispensing these units would be quite cumbersome for large scale operations because of the large number of Wheaton jars and test tubes involved. A submerged culture method would need to be developed for mass production of spores.

Several histological methods were tried to obtain micrographs that would depict the pathological and histopathological damage to the organs and tissues of the infected larvae. But because very little, if any histopathological studies have been done on internal structures of larval mosquitoes, or immature insects in general, this researcher's attempts in this area presented a challenging experience -- "fishing in muddy waters". Several different kinds of fixatives and stains were tried, and numerous histological methods were tried in an attempt to illustrate pathological and histological damage due to leptolegnial infection on two-day old larvae of Ae. triseriatus. The few techniques that yielded meaningful results were described in the experimental procedure section. The outcome of those techniques are illustrated

in the result section to serve as models for pathomorphological and pathohistological phenomena that occur in mosquito larvae infected with Leptolegnia sp.

The organs that suffered the major pathological changes were the anal papillae, the midgut, the trachea, the longitudinal muscles of the thorax and abdomen and the labial and pharyngeal muscles in the head, especially in the mouth. Selected tissues in these organs seemed to have been targeted for invasion and subsequent destruction. Although photographs and micrographs in corresponding treatment are not available for each of the observations made, it would be expedient to describe the observations with hope that such information may provide guidelines for future research. The destruction of the fatbody early in the infection process is suggestive of the sterol need stated by McInnis (1971) for Lagenidium sp.

Leptolegnial mycosis developed very quickly in young aedian larvae, resulting in the early death of the larvae, largely due to tissue destruction. McInnis and Zattau (1982) remarked that death of larvae infected by Leptolegnia sp. was probably due to tissue destruction or physiological starvations as suggested by Domnas et al. (1974) in relation to lagenidial mycosis, but they did not present documentation of damaged tissues as evidence for that remark. Tissue destruction as an ultimate cause of

death of infected larvae has also been suggested for other fungal pathogen of mosquitoes, McCray et al. (1973). In this study, illustrated evidence is presented to support the notion that infected larvae die as a result of physical or physiological damage to the tissues. Excessive vacuolation of the epithelial tissues, nuclear distortion and hypertrophy observed in this study from damage due to a toxin like reaction are similar to damages caused by toxic actions of Baccillus thuringiensis (Lahkim-Tsror et al. 1983, Percy and Fast 1883).

The observed engorgement of the midgut and thorax, the presence of encysted zoospore in these regions and the germination of zoospore cysts through the alimentary tract are all similar to the observation made by McInnis and Zattau (1982) describing infection of Ae. aegypti larvae by Leptolegnia sp. The visible engorgements of the thoracic and upper abdominal regions denote the presence of bacteria and zoospore cysts within the esophagus, gut, and hemocoel as seen in the micrographs (Figures 10, 11, 12, 14, 17, 35 and 36). The accumulation of bacteria and spores in the midgut either through mechanical action or through some enzyme or toxic productions, results in lysing and vacuolation of the cytoplasmic contents of epithelial tissues lining the gut, and distortion of the nuclei of cells that constitute these tissues (Figures 37). Similar

enzymatic or toxic action by invading zoospores resulted in destruction of the stomodael membrane (Figure 36B).

The whorls of annular structures and network of muscle and nerve fibers in the osmoregulatory organ, the anal papillae, are generally destroyed by what seems to be an enzymatic or toxic action of invading or encysted zoospores,resulting in gross disfiguration of the anal papilla (Figures, 33A and 34A). The teanidial bars of the trachea were destroyed by actions of invading spores and bacteria associated with them, and often a dark pigmented spot on the walls of the trachea denoted a wound reaction, the possible site of penetration of a zoospore. A similar notation of dark, pigmented spots was made by McCray et al. (1973) and McInnis and Zattau (1982) in the integuments of infected mosquito larvae. Tracheal blockage by a number of zoospores, as shown in Figures 13 and 18, often results in asphyxiation indicated by a change of color of the trachea noted under gross microscopic examination. Death by asphyxiation due to tracheal blockage or released of toxin into the hemocoel was described for mosquito species infected by terrestrial fungus Beauveria bassiana (Clark et al. 1968). Sweeney (1983) suspected toxin released into the hemocoel by penetrating hyphae of Culicinomyces invading the larvae of Anopheles hilli as contributing to the death of the larvae.

Leptolegnial mycosis started from the thorax and head regions of the infected larvae, followed by the upper abdominal region, where the midgut is located, preceding to the rectal area, anal papillae and the spiracular area of the larvae. The lower abdominal region where the hindgut is located is generally the last region to become infected. The observation that mycosis is initiated from the head and thorax is similar to reports by McCray et al. (1973), Washino (1981), Fetter-Lasko and Washino (1983) in reference to Lagenidium giganteum and McInnis and Zattau (1982) in reference to Leptolegnia sp. They also reported leptolegnial mycosis in the midgut, abdomen and anal papilla.

An investigation of leptolegniasis in the head of dead Ae. triseriatus larvae 12 hours after exposure showed that zoospores invade the labial (cranial) muscles arising from the prementum, and the pharyngeal (epipharyngeal) muscles behind the bucal cavity by perhaps secreting an enzyme which lyses the muscle fibers, leaving a lesion or clear zone in which the zoospore encyst, with a mass of mucilagenous substances around its body (Figure 37C). Wolf and Wolf (1947) noted enzyme production and positive chemotaxis as factors that aid penetration of pathogenic fungi into host tissues. The flattened epithelial layer which lines the pharyngeal cuticle is destroyed by encysted

and germinated spores which invade the layer. Remnants of electron dense nuclear materials denote cytological damage to the cells of the epithelial tissue by similar enzymatic mechanism, as described for the muscle tissue (Figure 37D). Snodgrass (1959) and Clements (1963) described, with illustrations, the functions of labial and pharyngeal apparati and larval feeding mechanics in mosquitoes.

It is likely, that by invading and damaging the these muscles, the zoospores interrupt the coordinated action of filtering materials in the bucal cavity and controlled pumping of filtrates into the adjacent esophagus. The symptomic gaping of the mouthparts described in the "mode of infection" section of this thesis, may be associated with the initial invasion of the pharyngeal muscle, and accumulation of spores, hyphogens and bacteria in the pharyngeal tract. Further investigation is needed to ascertain if the causal agent for that lesion is indeed enzyme or toxin, and the specific enzyme or toxin identified. Availability of such information might be helpful in determining a carrier substance for the formulation of leptolegnial spores, and also might provide a model for studying the possibility of a physiological resistance developing in the future.

An investigation of thoracic leptolegnia mycosis with TEM section of dead larva, 12 hours post inoculation showed

similar histopathological damage as described for the head. Masses of zoospores in different configurations, hyphogens and bacteria in the hemocoel of the thorax caused hyperplasia. There also appeared to be an increase in the fluid content of the thoracic cavity. This in conjunction with the hyperplasic condition, resulted in the engorgement of the thoracic region seen under gross examination. This finding correlates a report by McInnis and Zattau (1982) that an infected larva has an enlarged thorax from which infection is initiated.

The pathological and histopathological conditions in the abdomen of larvae killed by leptolegnial infection include damage to the epithelial tissues, destruction of the hemocytes leaving a dark area in the hemocoel and destruction of the fat bodies, leaving a dark matrix around a gray zone. Zoospores and bacteria often accumulate in the cavity of the peritrophic membrane of the midgut, causing in some diseased larvae, an extrusion of this membrane. Damage to the longitudinal muscle is also evident in figure 37H as described for other regions of the larval body. Lysing of fat bodies and hemocytes was observed, similar to the lysing of hemocytes of a lepidopterian larva by Nomuraea rileyi reported by Thorvilson et al. (1985).

The encysted zoospores and the developing sporangia aroused a defense reaction in the hemocoel of the mosquito larvae resulting in melanization of the spore and an apparent encapsulation of the sporangia (Figure 21) stopping further development of that spore bearing body. Similar melanization and encapsulation response was reported for Anopheles hilli and Culex quinquefasciatus larvae invaded by Culicinomyces clavisporus (Sweeney 1983) and larvae of Culiseta inornata infected by Coelomomyces psorophorae (Travland 1979b). An investigation into melanization and encapsulation processes in fungal pathogens at this early stage will aid in implementation of a control program that will counteract possible development of resistance in the host against the pathogen in the future.

SUMMARY

Leptolegnia sp., an aquatic, Oomycete pathogen of mosquito larvae, can be established on a half-split hemp seed, and this established culture used effectively for storage and for infecting several populations of two day old larvae of a tree hole mosquito, *Aedes triseriatus*. In this investigation, zoospores of *Leptolegnia sp.*, released from the split hemp seed inoculation unit, proved to be a very fast acting pathogen capable of killing 50% of the total larval population within 11.5 hours of exposure, at the rate of 78%-96% larvae within the first 24 hours of exposure, and eliminating the 100% of the larval population within 48-72 hours of exposure. The pathogenic rate exhibited by this pathogen follows a density dependent factor whereby, larval mortality is high under high density of host and pathogen.

The split hemp seed inoculation unit of *Leptolegnia sp.* remained persistent through 27 days in a water medium and released zoospores that were pathogenic to five sequential batches of mosquito larvae, inducing a mortality rate averaging 99% per batch within 72 hours of exposure. There was no statistically significant difference in the pathogenic rate and potential of the zoospores released

against the five batches of larval mosquitoes tested for persistent infection. In a choice study, a statistically significant number of motile zoospores of Leptolegnia sp. orientated to the homogenate of Aedes triseriatus larvae as a medium upon which they encysted and adhered for penetration.

Leptolegnia sp. established on split hemp seed and stored for 12 months at 5°C and 25°C did not lose viability and maintained a pathogenic potential capable of inducing an average of 90% mortality in the larval host. Though there was a statistically significant difference in the pathogenic rate of Leptolegnia sp. stored at 5°C and those stored at 25°C due to more hyphal death in the latter than the former, the over all pathogenic potential between these two groups did not change significantly. Longevity of Leptolegnia sp. stored in agar slants for 24 months at 5°C and 25°C was retained by culturing it on cornmeal dextrose and peptone (CMDP) agar and peptone yeast and glucose (PYG) agar either supplemented with 10% glycerol or covered with white mineral oil. The pathogenic potential of these stored slants was not reduced upon transferring the slant culture to cornmeal agar and establishing it on the split hemp seed inoculation unit.

The study on the mode of infection and some conditions affecting it, showed that the two-day old larvae of Aedes

triseriatus become infected by the zoospores of Leptolegnia sp. primarily through ingestion of the encysted and non-encysted spores and also by gnawing at the fungal hyphae and mycelia. A secondary mode of infection included penetration through the anal opening and also though the walls of the trachea via the hemocoel of the larvae. Penetration through the spiracular opening and integument were tertiary, occuring probably under conditions of stressed and weakened larvae. Food scarcity and availability did not statistically increase or decrease the rate at which pathogenicity occurred. Also, the length of time for which the host was exposed to the pathogen did not have a statistically significant effect on the rate or potential for pathogenicity.

Symptoms of leptolegnial infection on Ae. triseriatus larvae included cessation of feeding, excessive grooming, palpitation, excessive muscular contraction, gaping of mouthparts, change to a much darker color than the original, change in position, reduction in wiggling activities and eventual sluggishness. Larvae dead from leptolegnial infection first settled to the bottom of the container prior to floating, which occurred upon the emergence of a mass of hyphae through the larval body, 48-72 hours after exposure. The target organs for infection include the mouthparts, thorax, foregut-esophagus, crop,

gastric ceaca, midgut, trachea, anal papillae, rectum and hindgut. In the mouth parts, labial and pharyngeal muscles were a target for destruction by invading zoospores. Longitudinal muscles of the thorax and abdomen, and the stomodael membrane of the foregut were also destroyed by invading zoospores. Tissues that suffered the greatest damage were the epithelial tissues lining the digestive tract, the fat bodies in the hemocoel, the ganglionic structures of the gastric ceacum, the osmoregulatory structures of the anal papillae and the taenidial rings of the trachea. Light and transmission electron microscope studies showed cytological damage to the epithelial and muscular tissues. An apparent combat between the zoospores and the hemocytes resulted in destruction of the hemocytes, and melanization of some of the encysted zoospores and sporangia in the hemocoel.

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