# MORTALITY AND DEVELOPMENT OF *AEDES* LARVAE EXPOSED TO POTENTIAL NATURAL PATHOGENS *ASPERGILLUS NIGER, FUSARIUM OXYSPORUM,* AND *PYTHIUM ULTIMUM*

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A THESIS

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

Comparative Medicine and Integrative Biology - Master of Science

2013

## ABSTRACT

MORTALITY AND DEVELOPMENT OF *AEDES* LARVAE
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Survival and development of *Aedes triseriatus,* the mosquito vector of La Crosse Encephalitis, were found to be inhibited in the presence of dried leaves of certain populations of the sugar maple *Acer saccharum.* It is suspected that these effects are due to fungal contaminants carried on the leaf surface. Pure cultures of *Aspergillus niger* and *Fusarium oxysporum* were isolated from experimental containers in which these effects were observed. Pure cultures were used to challenge larvae of *Ae. triseriatus* and the invasive species *Aedes japonicus japonicus.* Results indicate that *Aspergillus* spores have a significant and repeatable lethal effect on larvae of the tested species, particularly those at the first and second instar. Results also indicate a significant inhibition of development in some experiments. Also implied is a possible competitive advantage of *Ae. j. japonicus* over *Ae. triseriatus* when exposed to spores of the fungus *Aspergillus*, as *Ae. j. japonicus* rates of survival were significantly higher than those of *Ae. triseriatus* when run concurrently.

## ACKNOWLEDGEMENTS

This work was funded by NIH grant # AI21884. I would like to thank Michigan State University and in particular the departments of Comparative Medicine and Integrative Biology, Entomology and Microbiology and Molecular Biology for support. This thesis would not have been possible without the constant support and guidance of my major advisor, Dr. Edward D. Walker and the members of my guidance committee: Dr. Michael Kaufman, Dr. Linda Mansfield, Dr. Terence Marsh, and Dr. A. Leonel Mendoza. I would also like to thank Drs. Vilma Yuzbasiyan-Gurkan and Victoria Hoelzer-Maddox for personal and program support, and of course, my friends, family, and coworkers for all of their advice and support throughout my entire academic career, and my husband Eric for everything he does every day.

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# CHAPTER 1: INTRODUCTION
# AND BACKGROUND INFORMATION

Mosquitoes vector a broad range of diseases of both human and veterinary medical importance. Among these are the arboviruses, such as the La Crosse Encephalitis Virus and West Nile Virus, which can cause severe neuroinvasive disease resulting in lasting neurological effects or death (Rust et al. 1999, Turell et al. 2001, Whitley and Gnann 2002, CDC 2009). As a result of their role in the transmission of these potentially devastating diseases, native and invasive mosquito species such as *Aedes triseriatus* and *Aedes japonicus japonicus* are of particular interest in current entomological research. It has been shown that various factors that affect the development of these mosquitoes during their immature stages can have a significant effect on the ability of the adults to successfully transmit disease (Grimstad and Haramis 1984, Grimstad and Walker 1991, Paulson and Hawley 1991), and that exposure to certain fungal species may have detrimental and/or fatal effects on developing larvae (Nnakumusana 1985, Moraes et al. 2001, Seye et al. 2009). Thus it is that the current research focuses on the effects of challenge with native fungal/oomycete genera on the larvae of treehole/container breeding mosquito species *Aedes triseriatus* and *Aedes japonicus japonicus.*

**Treehole environments.** As indicated by its common name, the Eastern Tree Hole Mosquito, *Aedes triseriatus* is a tree hole-dwelling species. Tree holes are, as the name implies, holes that form in the body of a tree and collect water. These can form in a variety of ways. Two of the main varieties are pans and rot holes. Pans, which tend to be shallow and have a solid bark base, form when parts of the tree, such as above-ground root sections, grow together and form depressions or pockets. These pans

collect water and insects, such as mosquitoes, subsequently utilize them for oviposition and larval development. Pans tend to be more ephemeral than deeper treeholes, such as rot holes. Rot holes form when a wound in the tree leads to the rotting away of tissue and eventual formation of a hole (Kitching 1971). These holes can be very deep or quite shallow, and the size of the opening may vary greatly, depending on the origin and development of the hole. Both pans and rot holes can collect a wide range of inputs such as leaves, soil, flowers, and decaying insect matter (Unpublished data). These inputs serve to increase the microbial community and dissolved nutrient content of the treehole, which in turn aids in and affects the development of larval insects such as mosquitoes (Fish and Carpenter 1982, Walker et al. 1991, Kaufman et al. 2010).

Relatively few studies have been conducted examining the fungal community typically found in tree holes. Several of those that have were conducted in beech-maple forests in the area near the campus of Michigan State University in East Lansing, MI (Kaufman et al. 2001, Yee et al. 2007, Kaufman et al. 2008). Interestingly, neither *Aspergillus* nor *Fusarium* species, which are the focus of this study, were among the 32 fungal species identified in the 2008 study by Kaufman, et al, which looked at bacterial and fungal species associated with leaf surfaces in naturally occurring tree holes (Kaufman et al. 2008) or the 2003 paper by Gönczöl and Révay which looked at hyphomycetous fungi in natural treehole microcosms in Hungary (Gonczol and Revay 2003). However, species within both genera have been isolated from tree holes containing larval mosquito habitats in Brazil (Luz et al. 2007).

***Aedes triseriatus* as a vector of disease.** *Aedes triseriatus* is most well-known as the primary vector of La Crosse Encephalitis (Sudia et al. 1971, Watts et al. 1972),

which is a viral infection caused by a virus within the family *Bunyaviridae.* It is a member of the California encephalitis group and, prior to the outbreak of West Nile Virus in 1999, was the causative agent behind the majority of identified cases of arboviral encephalitis in the United States (Rust et al. 1999, Whitley and Gnann 2002). It is still the cause of most cases of California serogroup neuroinvasive disease (CDC 2009), and has been shown to infect both humans and occasionally dogs, who can then transfer the disease back to owners/caretakers (Tatum et al. 1999).

La Crosse virus is transmitted by mosquitoes in the genus *Aedes*, specifically *Ae. triseriatus* and the invasive species *Ae. albopictus* in the United States (Watts et al. 1972, Gerhardt et al. 2001). The virus amplifies in small mammal hosts such as chipmunks, squirrels, and rabbits and is picked up by female mosquitoes when they take a blood meal (Gauld et al. 1974, Yuill 1984). After establishing an infection in the mosquito, the virus replicates and disseminates throughout the body of the mosquito. One to two weeks post-infected blood meal, the virus infects the salivary glands and amplifies. From here, it is passed to small mammal hosts during subsequent blood meals. In addition to this cross-species transmission, the virus can be passed directly to offspring via viral infection of the mosquito ovaries that then passes to the eggs (Borucki et al. 2002). This transovarial transmission allows the virus to survive the winter season in the protected environment of the mosquito egg (Watts et al. 1974). The La Crosse virus can also be passed from mosquito to mosquito via venereal transmission (Thompson and Beaty 1977).

La Crosse Encephalitis is rarely fatal. Rather, it usually manifests as a mild viral infection in humans, frequently without noticeable symptoms. When symptoms do

occur, they are typically mild and include fever, headache, fatigue, and nausea. The fact that the symptoms are so minor and generic leads to significant underreporting and/or misdiagnosing of La Crosse Encephalitis (McJunkin et al. 2001, CDC 2009). However, in the United States, approximately 80-100 cases each year develop into a severe, potentially fatal neuroinvasive disease characterized by seizures (CDC 2009) that closely resembles the disease caused by herpes simplex viral encephalitis (McJunkin et al. 1997, Sokol et al. 2001). These neuroinvasive infections occur when the virus, which replicates in muscle tissue, is able to reach a high enough viremia to penetrate the blood brain barrier and cause apoptosis in neural cells of the brain (Hollidge et al. 2010). Approximately 3,600 cases of neuroinvasive encephalitis caused by a member of the California encephalitis group (mostly La Crosse Encephalitis) were reported in the United States from 1964 to 2010. This severe presentation is most prevalent in children in the Eastern half of the U.S. and occurs between five and fifteen days after being bitten by an infected mosquito. Most cases resolve without lasting effects, but occasionally the disease does prove fatal (CDC 2009). In a small number of cases, lasting neurological effects such as cognitive impairment, attention deficit, and impaired voluntary movement may also develop. These can be transient and disappear after only a short time, or they may be permanent (McJunkin et al. 2001, Utz et al. 2005, CDC 2009, Hollidge et al. 2010). These neurologic after-effects can lead to severe impairment, changes in behavior and personality, disabilities, and social impairment. They may also cause significant hardship and emotional distress for the families of the affected children (Utz et al. 2005).

Recently, the range of La Crosse Encephalitis has been increasing, and more

cases are being reported in the Southeastern United States than have been historically (Jones et al. 1999, Nasci et al. 2000, CDC 2009). Unfortunately, awareness of this disease has not been increasing. There is a surprising lack of public awareness of this and related diseases (Soldan and Gonzalez-Scarano 2005), and Utz, *et al* (2005) found when studying this disease in North Carolina, that "LAC encephalitis cases have been reported from the mountains of NC since the mid 1960s […], yet the majority (80%) of families who participated in our study were not aware of the disease even though they resided in endemic areas."

In addition to its role in the transmission of the La Crosse Encephalitis Virus, *Ae. triseriatus* is a competent vector for several other diseases as well. This means that it is able to become infected by a virus or other pathogen, maintain and amplify the infection, and transmit said infection to naïve hosts. Of note, it has been shown that *Ae. triseriatus* could serve as an effective bridge vector for West Nile Virus based on its ability to become infected by feeding on infected chickens and its subsequent ability to transmit that infection to uninfected birds (Turell et al. 2005). However, given that the primary hosts of this mosquito species are small mammals such as chipmunks and squirrels, rather than the avian hosts which are vital to the West Nile Virus transmission cycle, its role in transmission of West Nile to humans is minimal at present. *Ae. triseriatus* is also a competent vector for Eastern Equine Encephalitis (Chamberlain et al. 1954) and the canine heartworm *Dirofilaria immitus* (Intermill 1973, Debboun et al. 2005), which has been shown to affect humans as both a pulmonary infection (Dashiell 1961, Merrill et al. 1980) and occasionally as an ocular infection (Moorhouse 1978, Avellis et al. 2011).

***Aedes japonicus* as a vector of disease.** In recent years, the mosquito *Aedes japonicus* has spread from its natural range in Asia to regions of North America and Europe (Peyton et al. 1999, Schaffner et al. 2009, Werner and Kampen 2013). *Ae. j. japonicus* has been shown to be a highly competent vector for West Nile Virus in the laboratory (Turell et al. 2001), even more so than *Ae. triseriatus*, as it was rated more highly as both an enzootic vector and a bridge vector (Turell et al. 2005). West Nile Virus has also been detected in wild populations of *Ae. j. japonicus* (Molaei et al. 2009, CDC 2012). Fortunately, while it may serve as an occasional bridge vector, its feeding habits are such that it is not likely to cause an immediate and dramatic increase in human cases of this disease, as it feeds primarily on mammals, rather than the bird species that are the amplifying vectors for West Nile Virus (Molaei et al. 2009). *Ae. j. japonicus* was also shown to develop disseminated infections of La Crosse Encephalitis Virus, which it could transmit, causing new infections in naïve mammal hosts (Sardelis et al. 2002b, Sardelis et al. 2002a). *Ae. j. japonicus* has also demonstrated an ability to transmit Eastern Equine Encephalitis to chickens in the laboratory (Sardelis et al. 2002b). However, the ability of a vector to transmit disease in the laboratory is not always indicative of its role in disease cycles in nature, as there are many more factors in play in the natural world than in most laboratory experiments.

**Vector development**. It is well established that factors affecting larval development have a subsequent effect on the physiology of the adult mosquito and the ability of adult mosquitoes to transmit arboviral diseases such as La Crosse Encephalitis. Larval nutrition plays a critical role in establishing the fitness and vectorial capacity of adult *Aedes* mosquitoes (Grimstad and Haramis 1984, Grimstad and Walker

1991). Additionally, competition is also a significant component to the development of mosquitoes that can act as competent vectors of disease as adults (Grimstad and Haramis 1984, Grimstad and Walker 1991, Paulson and Hawley 1991, Alto et al. 2005). Generally, mosquitoes that have experienced malnutrition or other stressors as larvae develop into smaller adults (Grimstad and Haramis 1984, Briegel 1990). These smaller adults have an increased capacity to transmit the La Crosse Encephalitis Virus. Early theories were that this increase in infection might be a result of the higher dose relative to body size when small adults were given a dose equivalent to that given to larger adults. However, subsequent research has shown that it is more likely due to enhanced dissemination of infection through the barrier of the midgut. In fact, the basement membrane of the midgut, which is one of several layers separating the contents of the midgut from the rest of the body, was found to be nearly twice as thick in smaller nutritionally deprived mosquitoes as it was in larger mosquitoes (McLintock 1978, Grimstad and Haramis 1984, Grimstad and Walker 1991, Paulson and Hawley 1991).

**Pathogenic and mosquitocidal effects of fungi and oomycetes on mosquito larvae.** There are many documented cases of fungi and oomycetes demonstrating pathogenic effects against mosquito larvae. For example, several members of the genus *Aspergillus* have been shown to be pathogenic to *Aedes, Anopheles,* and *Culex* mosquitoes (Nnakumusana 1985, Moraes et al. 2001, Seye et al. 2009). *Aspergillus clavatus* was shown to be effective at killing larvae of all three genera, though it was more effective against mosquitoes of the genus *Aedes* than against mosquitoes of the genus *Culex* (Seye et al. 2009). In another study, Moraes, *et al* (2001) tested eleven different strains of *Aspergillus* fungi against *Aedes* and *Culex* mosquitoes and found up

to 100% mortality, in some cases within 24 hours. The Moraes, et al study looked specifically at *Aspergillus flavus, Aspergillus kanagawaensis, Aspergillus ochraceus, Aspergillus sclerotiorum*, and *Aspergillus sulphureus*. These strains were tested against *Culex nigripalpus* and *Aedes fluviatilis* mosquitoes. In three tests, two with *Aedes* mosquitoes and one with *Culex*, mortality rates above 65% were observed within 24 hours.

Another group of fungi known to be pathogenic to immature mosquitoes is the collection of filamentous fungi within the genus *Fusarium* (Hasan and Vago 1972, Teetor-Barsch and Roberts 1983, Prakash et al. 2010). A classic study by Hasan and Vago (1972) showed that larvae exposed to conidia of the fungus *Fusarium oxysporum* reached between 51% and 83% mortality, with higher levels being noted in populations that had been injured prior to exposure. This pattern of spore invasion at mosquito wound sites has been found in other studies. In a study by Clark, et al (1966), for instance, an oomycete in the genus *Pythium* was found to invade *Aedes triseriatus* larvae preferentially at wound sites.

Studies indicate that both *Aspergillus* and *Fusarium* species can cause negative effects in mosquito larvae via multiple pathways. Species in both genera are known to produce mycotoxins (Govindarajan et al. 2005). Some species of *Aspergillus* produce aflatoxin, a highly potent □ycotoxins that has been shown to cause carcinogenic effects in vertebrates, particular in the liver (Alpert et al. 1971, Eaton and Gallagher 1994, Wang et al. 1996). Species in the genus *Fusarium* produce several types of mycotoxins, including the trichothecenes, the fumonisins, and zaeralenone (Dâ€™Mello et al. 1999, Placinta et al. 1999, Eriksen and Pettersson 2004). Extracellular filtrates of

cultures of both *Aspergillus* and *Fusarium* have been found to cause high mortality in mosquito bioassays utilizing the species *Culex quinquefasciatus* (Govindarajan et al. 2005). In addition, both *Aspergillus* and *Fusarium* can infect mosquito larvae directly. Invasion of the mosquito body cavity can occur through ingestion of spores, invasion at wound sites, or direct penetration of the larval cuticle (Hasan and Vago 1972, Teetor-Barsch and Roberts 1983, Moraes et al. 2001, Seye et al. 2009).

Despite the abundance of literature on interactions between mosquitoes and their fungal pathogens, very little information exists regarding what fungi have pathogenic effects on larvae of the treehole mosquito *Aedes triseriatus*. However, *Ae. triseriatus* larvae have been shown to be susceptible to several different genera of fungi and oomycetes. One such genus is *Coelomomyces,* a generalist pathogen that infects many different insects, but is found most often in mosquitoes (Anthony et al. 1971, Whisler et al. 1974, Couch and Bland 1985). Another important genus that has been shown to have significant pathogenic effect against *Ae. triseriatus* is *Lagenidium.* It is important to note that *Lagenidium* is an oomycete, not a true fungus (McCray Jr et al. 1973). In fact, *Lagenidium* was found to be so effective at killing mosquito larvae that it was approved in three formulations under the name Laginex for use as a larvicidal agent by the United States Environmental Protection Agency (EPA) in 1996. It was used in an aquatic suspension that was sprayed into natural larval habitats such as drainage sewers, tires, streams, and rice paddies (Kerwin and Washino 1988, USEPA 2001). In August of 2011, the EPA published a request from Agraquest to voluntarily cancel production and sales of Laginex (USEPA 2011).

**Table 1.1**. Fungal/oomycete genera known to be pathogenic to *Aedes triseriatus*.

| **Genus** | **Reference** |
|---|---|
| *Coelomomyces* | Couch and Bland, 1985 |
| *Funicularius* | Zaim *et al,* 1979 |
| *Lagenidium* | Couch, 1935, McCray, 1973 |
| *Leptolegnia* | Seymour, 1984 |
| *Metarhizium* | Andrade, 1993 |
| *Pleistophora* | Chapman, 1974 |
| *Pythium* | Clark *et al*, 1966, Nnakumusana 1985 |
| *Smittium* | Williams and Lichtwardt, 1972 |
| *Tolypocladium* | Nadeau and Boisvert, 1994 |
| *Verticillium* | Ballard and Knapp, 1984 |

A list of fungal/oomycete genera reported to be pathogenic to *Ae. triseriatus* can be found in Table 1. Notably, neither *Aspergillus* nor *Fusarium* appear to have been evaluated for activity against *Ae*. *triseriatus* larvae. As these are both fairly ubiquitous fungi, they are species that *Ae. triseriatus* could be reasonably expected to encounter in their natural habitat, so understanding more about the interactions between these species is desirable for expanding our base of knowledge regarding mosquito development in the wild.

The third potentially pathogenic genus utilized in this study, *Pythium,* is an oomycete genus typically associated with blights and wilts of plants (Martin and Loper 1999). However, some species are also pathogens of both vertebrate and invertebrate hosts. Most famous among the animal pathogens is the species *Pythium insidiosum*, which causes very serious, and often fatal, disease in vertebrates such as horses, dogs, cats, and humans, among others (Gaastra et al.). *P. insidiosum* has also recently been isolated from a mosquito in India (Schurko et al. 2003). Other species of *Pythium* have been reported as pathogens of mosquitoes as well. In fact, both Clark *et al* (1966) and Nnakumusana (1985) have shown *Pythium* spp. To be pathogenic to *Ae. triseriatus*,

though in both cases, identification is restricted to "*Pythium* sp." *Pythium guiyangense* has also been identified as a mosquito-pathogenic species (Su 2008).

**Objectives and Hypotheses**. Based on the above information, the following studies aim to further explore the relationship between *Aedes* larvae and potentially entomopathic fungal/oomycete genera *Aspergillus*, *Fusarium*, and *Pythium*. The rationale behind this set of studies was twofold: both to look at the basic science of the interactions between these naturally occurring species and to examine potential natural pathogens of disease-vectoring mosquito species in an attempt to identify fungi that may utilize chemicals or processes that could be exploited in future mosquito control agents. The objectives and major hypotheses are as follows:

**Objective 1:** Assess the presence and magnitude of mosquitocidal or inhibitory effects of isolated fungal/oomycete species against *Ae. triseriatus* larvae.

**Hypothesis 1:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant mosquitocidal effects when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 2:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant inhibition of growth and development when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 3:** Mosquitocidal effects will increase with mixed-species inoculum as compared with single-species inoculum.

**Objective 2:** Characterize the mode of action of mosquitocidal fungal/oomycete isolates against *Ae. triseriatus* larvae.

**Hypothesis 1:** Mortality and inhibition of development of *Ae. triseriatus* larvae challenged with *Aspergillus* and *Fusarium* isolates is due to a combination of secondary metabolites and direct infection of larvae.

**Hypothesis 2:** *Pythium* isolate effects are due to direct infection of mosquito larvae by motile zoospores, not secondary metabolites.

## LITERATURE CITED

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**Alpert, M. E., M. S. R. Hutt, G. N. Wogan, and C. S. Davidson. 1971.** Association between aflatoxin content of food and hepatoma frequency in Uganda. Cancer 28: 253-260.

**Alto, B. W., L. P. Lounibos, S. Higgs, and S. A. Juliano. 2005.** Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology 86: 3279-3288.

**Andrade, C. F. S. 1993.** *Metarhizium anisopliae* (Metsch.) Sorok.: Potential to recycle in mosquito larvae.

**Anthony, D. W., H. C. Chapman, and E. I. Hazard. 1971.** Scanning electron microscopy of the sporangia of species of Coelomomyces(Blastocladiales: Coelomomycetaceae). Journal of Invertebrate Pathology 17: 395-403.

**Avellis, F. O., A. Bartolino, P. Benedetti, L. H. Kramer, P. Mora, and F. Rivasi. 2011.** A case of human conjunctival dirofilariosis by Dirofilaria immitis in Italy. Vector-Borne and Zoonotic Diseases 11: 451+.

**Ballard, E., and F. Knapp. 1984.** Occurrence of the fungus Verticillium lecanii on a new host species: Aedes triseriatus (Diptera: Culicidae). Journal of Medical Entomology 21: 751-751.

**Briegel, H. 1990.** Metabolic relationship between female body size, reserves, and fecundity of Aedes aegypti. Journal of Insect Physiology 36: 165-172.

**CDC. 2009.** La Crosse Encephalitis. *In* C. f. D. C. a. Prevention [ed.], Atlanta, GA.

**CDC. 2012.** Mosquito species in which West Nile virus has been detected, United States, 1999-2012.

**Chamberlain, R. W., R. K. Bikes, D. B. Nelson, and W. D. Sudia. 1954.** Studies on the North American Arthropod-borne Encephalitides. American Journal of Epidemiology 60: 278-285.

**Chapman, H. C. 1974.** Biological control of mosquito larvae. Annual Review of Entomology 19: 33-59.

**Clark, T. B., W. R. Kellen, J. E. Lindegren, and R. D. Sanders. 1966.** Pythium sp. (Phycomycetes:Pythiales) pathogenic to mosquito larvae. Journal of Invertebrate Pathology 8: 351-354.

**Couch, J. N., and C. E. Bland. 1985.** The genus Coelomomyces. Academic Press.

**D'Mello, J. P. F., C. M. Placinta, and A. M. C. Macdonald. 1999.** Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology 80: 183-205.

**Dashiell, G. F. 1961.** A Case of Dirofilariasis Involving the Lung. The American journal of tropical medicine and hygiene 10: 37-38.

**Debboun, M., T. J. Green, L. M. Rueda, and R. D. Hall. 2005.** Relative Abundance Of Tree Hole-Breeding Mosquitoes In Boone County, Missouri, USA, With Emphasis On The Vector Potential Of Aedes Triseriatus For Canine Heartworm, Dirofilaria Immitis (Spirurida: Filariidae) 1. Journal of the American Mosquito Control Association 21: 274-278.

**Eaton, D. L., and E. P. Gallagher. 1994.** Mechanisms of Aflatoxin Carcinogenesis. Annual Review of Pharmacology and Toxicology 34: 135-172.

**Eriksen, G. S. I., and H. Pettersson. 2004.** Toxicological evaluation of trichothecenes in animal feed. Animal Feed Science and Technology 114: 205-239.

**Gaastra, W., L. J. A. Lipman, A. W. A. M. De Cock, T. K. Exel, R. B. G. Pegge, J. Scheurwater, R. Vilela, and L. Mendoza.** Pythium insidiosum: An overview. Veterinary microbiology 146: 1-16.

**Govindarajan, M., A. Jebanesan, and D. Reetha. 2005.** Larvicidal effect of extracellular secondary metabolites of different fungi against the mosquito, Culex quinquefasciatus Say. Tropical biomedicine 22: 1-3.

**Grimstad, P. R., and L. D. Haramis. 1984.** Aedes triseriatus (Diptera: Culicidae) and La Crosse virus III. Enhanced oral transmission by nutrition-deprived mosquitoes. Journal of Medical Entomology 21: 249-256.

**Grimstad, P. R., and E. D. Walker. 1991.** Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and transmission. Journal of Medical Entomology 28: 378-386.

**Hasan, S., and C. Vago. 1972.** The pathogenicity of Fusarium oxysporum to mosquito larvae. Journal of Invertebrate Pathology 20: 268-271.

**Intermill, R. 1973.** Development of Dirofilaria immitis in Aedes triseriatus Say. Mosquito News 33: 176-181.

**Jones, T. F., A. S. Craig, R. S. Nasci, L. E. Patterson, P. C. Erwin, R. R. Gerhardt, X. T. Ussery, and W. Schaffner. 1999.** Newly recognized focus of La Crosse encephalitis in Tennessee. Clinical infectious diseases 28: 93-97.

**Kerwin, J. L., and R. K. Washino. 1988.** Field Evaluation of Lagenidium giganteum (Oomycetes: Lagenidiales) and Description of a Natural Epizootic Involving a New Isolate of the Fungus. Journal of Medical Entomology 25: 452-460.

**Martin, F. N., and J. E. Loper. 1999.** Soilborne plant diseases caused by Pythium spp.: ecology, epidemiology, and prospects for biological control. Critical Reviews in Plant Sciences 18: 111-181.

**McCray Jr, E., C. Umphlett, and R. Fay. 1973.** Laboratory studies on a new fungal pathogen of mosquitoes. Mosquito News 33: 54-60.

**McJunkin, J. E., R. Khan, C. Emily, D. L. Parsons, L. L. Minnich, R. G. Ashley, and T. F. Tsai. 1997.** Treatment of severe La Crosse encephalitis with intravenous ribavirin following diagnosis by brain biopsy. Pediatrics 99: 261-267.

**McJunkin, J. E., E. C. Reyes, J. E. Irazuzta, M. J. Caceres, R. R. Khan, L. L. Minnich, K. D. Fu, G. D. Lovett, T. Tsai, and A. Thompson. 2001.** La Crosse Encephalitis in Children. New England Journal of Medicine 344: 801-807.

**Merrill, J. R., J. Otis, W. D. Logan Jr, and M. B. Davis. 1980.** The Dog Heartworm (*Dirofilaria immitis*) in Man. Journal of the American Medical Association 243: 1066-1068.

**Molaei, G., A. Farajollahi, J. J. Scott, R. Gaugler, and T. G. Andreadis. 2009.** Human Bloodfeeding by the Recently Introduced Mosquito, Aedes japonicus japonicus, and Public Health Implications. Journal of the American Mosquito Control Association 25: 210-214.

**Moorhouse, D. 1978.** Dirofilaria immitis: A cause of human intra-ocular infection. Infection 6: 192-193.

**Moraes, A., G. Costa, M. Barcellos, R. Oliveira, and P. Oliveira. 2001.** The entomopathogenic potential of Aspergillus spp. in mosquitoes vectors of tropical diseases. J. Basic Microbiol 41: 45-49.

**Nadeau, M., and J. Boisvert. 1994.** Larvicidal activity of the entomopathogenic fungus Tolypocladium cylindrosporum (Deuteromycotina: Hyphomycetes) on the mosquito Aedes triseriatus and the black fly Simulium vittatum (Diptera: Simuliidae). Journal of the American Mosquito Control Association 10: 487-491.

**Nasci, R., C. Moore, B. Biggerstaff, N. Panella, H. Liu, N. Karabatsos, B. Davis, and E. Brannon. 2000.** La Crosse encephalitis virus habitat associations in Nicholas County, West Virginia. Journal of Medical Entomology 37: 559-570.

**Nnakumusana, E. 1985.** Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to Aspergillus parasiticus and its effects on fecundity and longevity of mosquitoes exposed to sporal infections in larval stages. Current Science 54: 1221.

**Paulson, S. L., and W. A. Hawley. 1991.** Effect of body size on the vector competence of field and laboratory populations of *Aedes triseriatus* for La Crosse Virus. Journal of the American Mosquito Control Association 7: 170-175.

**Peyton, E., S. R. Campbell, T. M. Candeletti, M. Romanowski, and W. J. Crans. 1999.** Aedes (Finlaya) japonicus japonicus (Theobald), a new introduction into the United States. DTIC Document.

**Placinta, C. M., J. P. F. D'Mello, and A. M. C. Macdonald. 1999.** A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. Animal Feed Science and Technology 78: 21-37.

**Prakash, S., G. Singh, N. Soni, and S. Sharma. 2010.** Pathogenicity of Fusarium oxysporum against the larvae of Culex quinquefasciatus (Say) and Anopheles stephensi (Liston) in laboratory. Parasitology research 107: 651-655.

**Rust, R. S., W. H. Thompson, C. G. Matthews, B. J. Beaty, and R. W. Chun. 1999.** Topical Review: La Crosse and Other Forms of California Encephalitis. Journal of child neurology 14: 1-14.

**Sardelis, M. R., M. J. Turell, and R. G. Andre. 2002a.** Laboratory Transmission of La Crosse Virus by Ochlerotatus j. japonicus (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

**Sardelis, M. R., D. J. Dohm, B. Pagac, R. G. Andre, and M. J. Turell. 2002b.** Experimental Transmission of Eastern Equine Encephalitis Virus by Ochlerotatus j. japonicus (Diptera: Culicidae). Journal of Medical Entomology 39: 480-484.

**Schaffner, F., C. Kaufmann, D. Hegglin, and A. Mathis. 2009.** The invasive mosquito Aedes japonicus in Central Europe. Medical and Veterinary Entomology 23: 448-451.

**Schurko, A., L. Mendoza, A. W. A. M. de Cock, and G. R. Klassen. 2003.** Evidence for geographic clusters: Molecular genetic differences among strains of Pythium insidiosum from Asia, Australia and the Americas are explored. Mycologia 95: 200-208.

**Seye, F., O. Faye, M. Ndiaye, E. Njie, and J. M. Afoutou. 2009.** Pathogenicity of the fungus, Aspergillus clavatus, isolated from the locust, Oedaleus senegalensis, against larvae of the mosquitoes Aedes aegypti, Anopheles gambiae and Culex quinquefasciatus. Journal of Insect Science 9.

**Seymour, R. L. 1984.** Leptolegnia chapmanii, an oomycete pathogen of mosquito larvae. Mycologia: 670-674.

**Sokol, D. K., M. B. Kleiman, and B. P. Garg. 2001.** LaCrosse viral encephalitis mimics herpes simplex viral encephalitis. Pediatric neurology 25: 413-415.

**Soldan, S. S., and F. Gonzalez-Scarano. 2005.** Emerging infectious diseases: the Bunyaviridae. Journal of neurovirology 11: 412-423.

**Su, X. 2008.** A Report of the Mosquito Host Range of Pythium guiyangense Su.

**Sudia, W., V. Newhouse, C. Calisher, and R. Chamberlain. 1971.** California group arboviruses: Isolations from mosquitoes in North America. Mosq. News 31: 576-600.

**Tatum, L. M., J. M. Pacy, K. S. Frazier, J. F. Weege, C. A. Baldwin, G. A. Hullinger, G. D. Bossart, and N. H. Altman. 1999.** Canine LaCrosse viral meningoencephalomyelitis with possible public health implications. Journal of Veterinary Diagnostic Investigation 11: 184-188.

**Teetor-Barsch, G. H., and D. W. Roberts. 1983.** Entomogenous Fusarium species. Mycopathologia 84: 3-16.

**Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001.** Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. Journal of Medical Entomology 38: 130-134.

**Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. O'guinn, T. G. Andreadis, and J. A. Blow. 2005.** An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 42: 57-62.

**USEPA. 2001.** *Lagenidium giganteum* (129084) Fact Sheet. *In* U. S. E. P. Agency [ed.].

**Utz, J. T., C. S. Apperson, and E. J. Dietz. 2005.** Social impacts of la crosse encephalitis in North Carolina. Human organization 64: 135-146.

**Wang, L.-Y., M. Hatch, C.-J. Chen, B. Levin, S.-L. You, S.-N. Lu, M.-H. Wu, W.-P. Wu, L.-W. Wang, Q. Wang, G.-T. Huang, P.-M. Yang, H.-S. Lee, and R. M. Santella. 1996.** Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. International Journal of Cancer 67: 620-625.

**Watts, D. M., C. D. Morris, R. E. Wright, G. R. DeFoliart, and R. P. Hanson. 1972.** Transmission of LaCrosse Virus (California Encephalitis Group) by the mosquito *Aedes triseriatus*. Journal of Medical Entomology 9: 125-127.

**Werner, D., and H. Kampen. 2013.** The further spread of Aedes japonicus japonicus (Diptera, Culicidae) towards northern Germany. Parasitology research 112: 3665-3668.

**Whisler, H. C., S. L. Zebold, and J. A. Shemanchuk. 1974.** Alternate host for mosquito parasite Coelomomyces. Nature 251: 715-716.

**Whitley, R. J., and J. W. Gnann. 2002.** Viral encephalitis: familiar infections and emerging pathogens. The Lancet 359: 507-513.

**Williams, M. C., and R. W. Lichtwardt. 1972.** Infection of Aedes aegypti larvae by axenic cultures of the fungal genus Smittium (Trichomycetes). American Journal of Botany: 189-193.

**Zaim, M., K. Baker, and H. Newson. 1979.** Natural infection of the tree hole breeding mosquito Aedes triseriatus (Diptera: Culicidae) with the fungus Funicularius triseriatus. Journal of Invertebrate Pathology 34: 199-202.

# CHAPTER 2: OBSERVATION OF INCREASED MORTALITY OF *AEDES TRISERIATUS* LARVAE EXPOSED TO POTENTIAL ENVIRONMENTAL CONTAMINANTSAND CHARACTERIZATION OF POTENTIAL ETIOLOGIC AGENTS *ASPERGILLUS NIGER* AND *FUSARIUM OXYSPORUM*

## Introduction

*Aedes triseriatus*, the Eastern Treehole Mosquito, is the primary vector for LaCrosse Encephalitis and competent to vector several other diseases, including the canine heartworm *Dirofilaria immitus* (Intermill 1973) and West Nile Virus (Turell et al. 2005). Due to its potential for disease transmission, and its prevalence in Southern Michigan, as well as its ability to survive and even thrive in established laboratory conditions, it is an ideal organism for laboratory testing and evaluation of larval development and factors affecting development.

In the laboratory, mosquito colonies are generally raised on a standardized diet. Often the nutrients necessary for growth are supplied via dried and powdered compounds such as liver powder (Arrivillaga and Barrera 2004), yeast (Rahuman et al. 2008), or ground pet foods, such as dog chow or fish food (Naksathit et al. 1999, Onken et al. 2004). However, during experimental procedures with treehole mosquitoes, it is common to see dried leaves used to encourage microbial growth as a nutrient source, to more closely mimic the conditions that would be seen in the mosquito's natural habitat (Fish and Carpenter 1982). This does add a level of complexity to an experimental container, as it is difficult to anticipate what microbes, fungal spores, etc, may be carried into the container on the surface of the leaves. In some cases, these contaminants can be incredibly detrimental to the development of the mosquitoes being reared, and may cloud experimental results. Such was the case with a recent experiment, during which dried leaves of the sugar maple *Acer saccharum* were used as a microbial substrate and a subsequent growth of filamentous material in the water

column and on the leaf surface seemed to have a dramatic inhibitory and lethal effect on developing larvae of the mosquito *Aedes triseriatus.*

The sudden and significant die-off of mosquitoes in the laboratory is a surprising phenomenon, and one that with further exploration might yield information that could be used to develop improved methods of control for these disease-carrying organisms. As such, this observational study aimed to identify and characterize potential causative agents of this effect.

## Materials and Methods

**Mosquito Culture.** The mosquitoes used for this work are *Aedes triseriatus,* Walton strain, and *Aedes japonicus japonicus*. Larvae are maintained in laboratory culture and provided ground Tetramin brand fish food (Tetra Werke, Melle, Germany) as a nutrient source. Laboratory colonies are regularly fed bovine blood (Hemostat Laboratories, Dixon, CA) via an artificial feeder made of blown glass. *Ae. j. japonicus* eggs were obtained from Dr. Linda McCuiston at Rutgers University in New Brunswick, New Jersey. Unless otherwise stated, larvae used for all experiments were less than 24 hours old and hatched in the laboratory.

**Initial Observations and Analysis.** It was observed during an unrelated experiment designed to evaluate the toxicity of ionic copper to larval *Aedes triseriatus* that first instar larvae of this mosquito species unexpectedly demonstrated dramatically increased levels of mortality regardless of treatment group (unpublished data). This increased mortality appeared to be associated with the use of dried leaves of the sugar

maple *Acer saccharum* as a source of nutrients in the experimental microcosms. Additionally, there was a distinct inhibition of development observed in those larvae that did survive to the end of the experimental timecourse. Surviving larvae appeared significantly smaller and underdeveloped when compared to larvae of the same age in previous experiments and in the laboratory colony. Microcosms showing this increase in mortality all had visible filamentous growth associated with the leaf surface and water column. To facilitate further analysis of the suspected contaminating factor, two 50 mL water samples were taken from each experimental microcosm. DNA was extracted from one sample per microcosm using the MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA was then amplified via PCR using primers targeting the 18S region of the fungal genome (Forward: 5'- TTA GCA TGG AAT AAT RRA ATA GGA -3', Reverse: 5'- ATT GCA ATG CYC TAT CCC CA -3') (Borneman and Hartin 2000). Thermocycler program was as indicated in Table 2.1. This gene fragment was cloned into *E. coli* using the pGEM T-easy Vector system (Promega; Madison, WI). The cloned region was then sequenced and used for BLAST searches of the GenBank database.

**Table 2.1.** Thermocycler program for fungal 18s PCR.

| Stage | Stage 1 | Stage 2 | | | Stage 3 | |
|---|---|---|---|---|---|---|
| Cycles | x1 | x30 | | | x1 | |
| Temp (°C) | 94.0 | 94.0 | 58.0 | 72.0 | 72.0 | 4.0 |
| Time | 2:00 | 0:45 | 0:30 | 1:00 | 7:00 | infinity |

**Ergosterol Analysis.** Ergosterol is one of the major components of the fungal cell membrane and is a well-established biomarker for evaluating the amount of live fungal biomass in a sample (Seitz et al. 1979). Ergosterol analysis was conducted as in

previous studies (Kaufman et al. 2001, Kaufman et al. 2002). Briefly, ergosterol was extracted from 50 mL water samples as follows: Samples were centrifuged to concentrate fungal biomass and pellets were resuspended in 5 mL of HPLC grade methanol. To this suspension, 2 mL of a 4% potassium hydroxide in 95% ethanol solution were added and samples were heated in an 80° C water bath for 30 minutes. Samples were cooled and deionized water (2 mL) was added. Hexanes (4 mL) were added to this solution and the sample was mixed thoroughly, then allowed to settle. After a clear bilayer formed, the upper layer, containing hexanes and extracted ergosterol, was removed and transferred to a new vial. To this vial, 3 mL hexanes were added and another bilayer was allowed to form. Again, the upper layer was removed and transferred to the same vial as the previous hexanes solution. This hexane/ergosterol solution was dried under nitrogen in a 40° C sand bath and the dried ergosterol was resuspended in methanol. This ergosterol solution was run through a Shimadzu Prominence UFLC (Shimadzu Scientific Instruments, Columbia, MD) unit and analyzed using the LCSolution software (Shimadzu Scientific Instruments, Columbia, MD) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

**Confirmation of Effect.** To confirm that the observed phenomenon was not an isolated incident, a limited bioassay was conducted. For this bioassay, White Oak (*Quercus alba*) leaves were used as a control. These leaves had been used extensively in previous experiments and shown to promote healthy growth and development of mosquito larvae. There was no documented evidence of increased mortality associated with these leaves. Experimental microcosms were constructed as follows: 345 mL milli-Q filtered water, 1 g dried maple or oak leaf (depending on

treatment group), and 5 mL treehole inoculum (water/debris collected from a natural treehole and homogenized in a lab blender) were combined in a 16 oz clear plastic food storage container (Figure 2.1). Four microcosms each were set up for treatment and control groups. Microcosms were allowed to sit one week at room temperature, covered with vented lids, to develop a robust microbial community prior to larval addition. After one week, 20 first instar *Ae. triseriatus* larvae were added to each microcosm. Larvae were maintained in the microcosms for 15 days, at which point the experiment was terminated due to larval mortality. For the confirmation of effect, a t-test with Welch's correction for unequal variances between groups was run. To examine the distribution of developmental stages between the two groups, a Wilcoxon Rank Sum test was run. All statistics were completed in Program R (R Foundation for Statistical Computing, Wien, Austria).

**Figure 2.1** Experimental Microcosms. (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.)

**Oomycete analysis**. Due to the conflict between the copious amounts of filamentous growth in experimental microcosms and the low ergosterol levels returned on analysis, it was determined that analyses should be run to survey for the presence of oomycetes in the experimental containers. Oomycetes could potentially explain these conflicting data, as they look and behave like fungus, but do not produce ergosterol, instead relying on exogenous cholesterol in the construction of cell membranes (McCorkindale et al. 1969, Gaulin et al. 2010) DNA was extracted using the MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) and amplified at the ITS1/5.8s region using *Pythium* specific primers (Forward: 5'-GAA GGA TCA TTA CCA CAC-3', Reverse: 5'-TAC GGA CAC TGA TAC AG-3', (Geraats et al. 2002))and thermocycler conditions as outlined in Table 2.2. This gene fragment was cloned into *E. coli* using the pGEM T-easy Vector system (Promega; Madison, WI). The cloned region was then sequenced and used for BLAST searches of the GenBank database.

**Table 2.2.** Thermocycler program for oomycete ITS1/5.8S PCR.

| Stage | Stage 1 | Stage 2 | | | Stage 3 | |
|---|---|---|---|---|---|---|
| Cycles | x1 | x30 | | | x1 | |
| Temp (°C) | 94.0 | 94.0 | 52.0 | 72.0 | 72.0 | 4.0 |
| Time | 1:00 | 0:30 | 0:30 | 1:00 | 7:00 | infinity |

**Natural Microcosm Survey.** In order to confirm that the isolated fungi are present in naturally occurring larval habitats, a survey of habitats on and around the campus of Michigan State University was conducted. Survey sites included treeholes and ponds on the main campus of Michigan State University, as well as in two woodlots

south of campus, Toumey and Hudson Woodlots. Water samples of 50 mL each were collected from 24 known or potential larval mosquito habitats, including natural treeholes, tires, and ornamental ponds. Polymerase chain reactions and gel electrophoresis were conducted with fungal and oomycete primers as described above.

**Maple Survey.** In order to determine whether the observed effect was generally associated with leaves of the sugar maple *Acer saccharum* or specific to leaves found in the same location as the original collection, a survey of maple leaves from select locations was conducted. Maple leaves were collected at the same location on the Michigan State University campus as the original contaminated stock, from both the ground and directly from the tree. This dual collection was to determine whether contamination from the soil or grass might be responsible for the observed effect. Leaves were also collected from a tree and the surrounding ground in one location in Hudson Woodlot south of campus. White Oak (*Quercus alba*) and Beech (*Fagus sp.*) leaves were used as controls, as both have been shown to consistently facilitate rapid, healthy growth of *Ae. triseriatus* larvae. These leaves were used in experimental microcosms set up as previously, with 345 mL milli-Q filtered water, 1 g dried leaf, and 5 mL treehole inoculum. Twelve microcosms were set up for each experimental group (Campus Ground, Campus Tree, Woodlot Ground, Woodlot Tree, Beech Control, Oak Control). After one week, the experiment was ended. Larvae were preserved in 70% ethanol. Mortality and developmental stage of preserved larvae were recorded and 50 mL water samples were taken for further analysis. Non-parametric statistical analyses were run to analyze these data. These tests included a Wilcoxon Rank Sum test

comparing mean mortality values, and Kruskal-Wallis and Mann-Whitney tests comparing the distribution of developmental stage.

**Fungal Isolations.** *Aspergillus* cultures were isolated as follows. Samples taken from suspect maple leaves were plated on Czapac Yeast Agar (CYA) to look for and isolate both epiphytic and endophytic fungi. For epiphytic fungi, intact leaf surfaces approximately equivalent in area to a 13 mm diameter leaf disc were swabbed with a sterile cotton-tipped applicator dipped in milli-Q filtered water. The cotton tip was then placed in 1 mL milli-Q water, swirled gently and allowed to soak for one minute. After one minute, the cotton was removed and 200 µL water was spread on CYA plates. Plates were incubated at room temperature. For endophytic fungi, two 13 mm leaf discs were cut using a standard cork borer and surface sterilized as follows; Leaf discs were soaked for 60 seconds in 70% ethanol, transferred to 75% commercial bleach for three minutes, transferred to a new beaker of 70% ethanol and rinsed thoroughly in milli-Q water (Lodge et al. 1996). Discs were then transferred to CYA plates and incubated at room temperature. After eight days, selected colonies were passed to new CYA plates containing chloramphenicol and incubation was continued at room temperature. After 14 additional days, spores of selected cultures were picked from individual fruiting bodies under a dissecting scope using a sterile probe and transferred to new CYA plates containing chloramphenicol and kanamycin to yield pure colonies of sampled fungi.

*Fusarium* cultures were isolated via a modification of the protocol by Ho and Ko (Ho and Ko 1997). Briefly, three pieces were cut from an actively growing culture, approximately 5 mm x 5 mm in size. These culture pieces were transferred to 20 mL of

sterile water in a standard 100 mm petri plate and allowed to sit for 48 hours at room temperature (24° C). After 48 hours, water cultures were transferred to a 4° C refrigerator and allowed to sit for 15 minutes. After 15 minutes, the chilled liquid culture was transferred to a 50 mL polypropylene centrifuge tube (Corning Incorporated, Tewksbury, MA) and vortexed for one minute. Plates of 2% water agar had been previously divided into 60 equally-sized squares, marked on the bottom of the plate. A predetermined amount of the chilled and agitated spore solution was added to each square. One full plate was inoculated with 0.25 µL per square. A second full plate was inoculated with 0.5 µL per square. Inoculated plates were incubated for 24 hours at room temperature. After 24 hours, each square of the grid was examined to evaluate spore presence and/or germination. Eight sections containing a single germinated spore were selected, carefully cut from the original culture plate, and transferred to new 2% water agar plates. These plates were monitored for growth and healthy colonies from seven of eight plates were passed to new plates after two weeks. One successful culture was selected for future use and continually passed on water agar and CYA media. Media recipes are given in Chapter 3.

**Identification.** Isolated colonies were identified using both morphological characterization and molecular analysis. Morphological observations were made using microscopic analysis of plated culture material, slide culture techniques, and digital imaging/measurement of characters such as hyphal width, sexual structures, and septation, as well as measurements of average growth rate on selected media. Molecular analysis was via PCR and sequencing of either the 18S or ITS regions, as described previously, followed by a BLAST search of resulting sequences.

## Results

**Initial Observations and Analysis.** Analysis of 280 cloned samples containing DNA extracted from initially contaminated water samples yielded mostly sequences from the genus *Aspergillus*. The most common results were *A. niger* and *A. awamori*. Subsequent analysis of purified spore solutions confirmed the identification as *Aspergillus niger*.

**Ergosterol Analysis.** Ergosterol samples from tested containers showed very low values (Averages: 0.035008 µg/leaf disc, standard deviation 0.03516, and 0.006479 µg/mL water, standard deviation 0.003531, n=94 in both cases), despite the copious visible growth in the containers.

**Confirmation.** After one week of growth, there was no noticeable difference between the appearance and activity level of larvae grown in maple and oak microcosms. However, by Day 12 of larval growth, there was a marked difference between the two treatment groups. There was a massive die-off of larvae in maple containers, and the few surviving larvae appeared small and sluggish, whereas the larvae in oak containers were large and very active. One oak container appeared to have more filamentous growth and smaller larvae than the others in its treatment group. On Day 13 of larval growth, deceased specimens were examined under a dissecting scope. A few were found to have extensive microbial growth on the anal papillae. By Day 14, only one of 80 larvae grown in maple microcosms was still alive. The experiment was ended on Day 15 and water samples were taken for ergosterol, DNA, and culture. Larvae were counted, sorted by instar, and preserved in 70% Ethanol.

Larval counts and distribution are given in Figures 2.2 and 2.3. The average survival in untreated controls was 79%, and in treatment groups was 1.25%. Statistical analysis showed that survival was significantly different between the two treatment groups (t-test, p=0.001563). A Wilcoxon rank sum test also showed that the distribution of larvae among developmental stages was significantly different between the two groups ($p < 2.2 \times 10^{-16}$), with the maple-based microcosms demonstrating a much higher proportion of early instars, implying that there is a significant inhibition of development when contaminated maple leaves are used in laboratory microcosms.

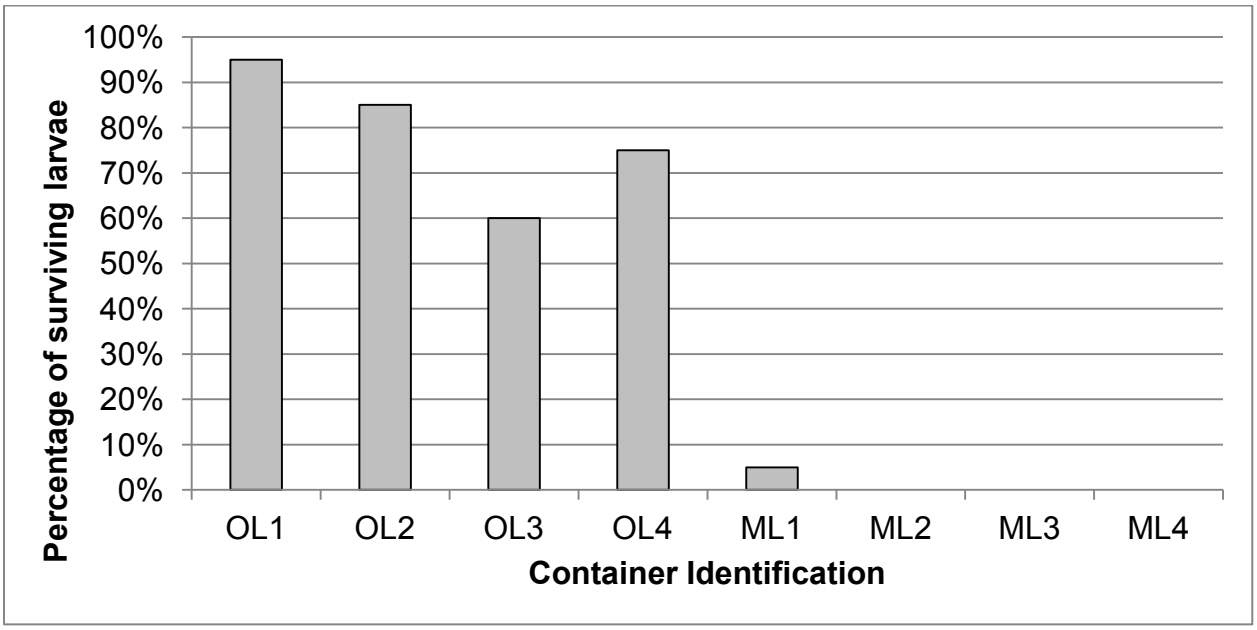


**Figure 2.2**. Larval survival after 15 days growth on oak or maple leaves.
Note: OL= Oak microcosm, ML=maple microcosm.

**Oomycete analysis**. Analysis of extracted DNA from initial microcosms showed a strong presence of sequences within the genus *Pythium*. The most frequent results were *P. sterilum, P. litorale,* and *P. delawarii*.

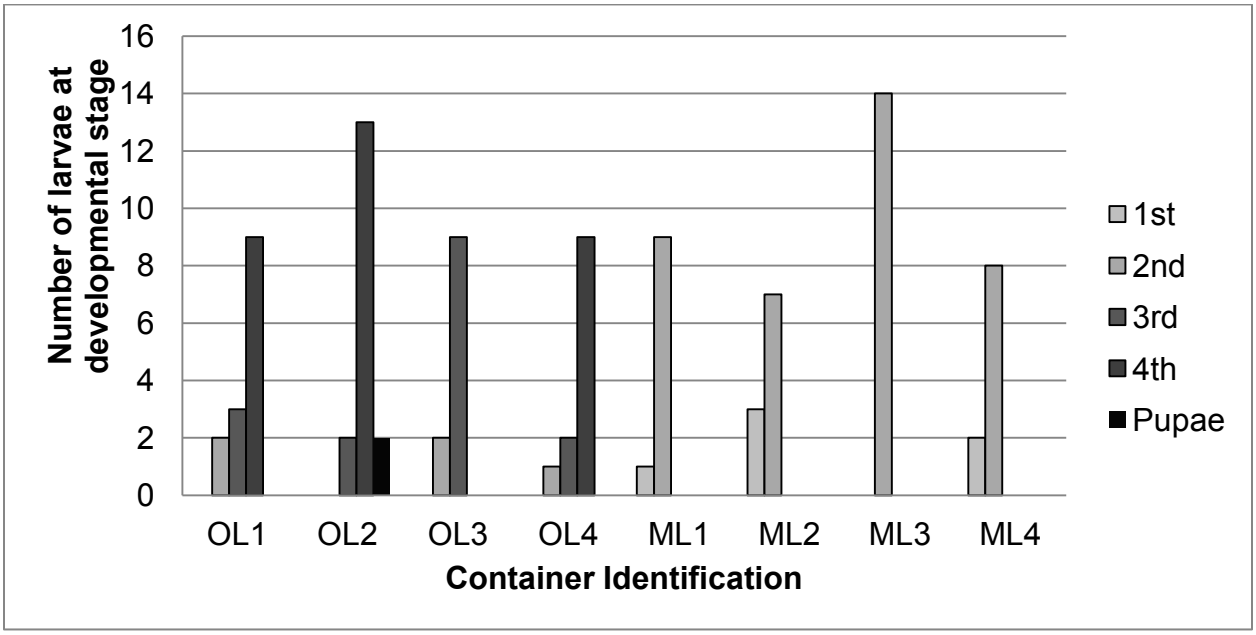


**Figure 2.3**. Development of larvae after 15 days growth on oak or maple leaves. Note the pronounced difference in general developmental progress between oak and maple microcosms. Light grey indicates early (first and second) instars, dark grey indicates late (third and fourth) instars, black indicates pupae.

**Surveys.** All natural samples produced positive amplicons for both *Pythium* and fungal DNA in PCR analysis. The maple survey showed a significant difference in effect based on location. Wilcoxon rank sum tests showed that larvae grown with leaves collected from the ground around the campus-located tree showed a much higher level of mortality at the end of two weeks than those grown with leaves either directly from

the tree ($p < 0.001$) or from Hudson Woodlot ($p < 0.001$). Kruskal-Wallis and Mann-Whitney tests on developmental stage showed a significant effect of leaf source on larval development, with “Campus Ground” leaves producing a much higher proportion of larvae at early developmental stages (first and second instars) than other maple sources, which produced larvae that had nearly all reached the third and fourth instars, and a small number of pupae ($p < 0.001$). The other maple collections did not differ significantly from the beech or oak control microcosms in mortality (Kruskal-Wallis, $p > 0.5$). Developmental stages were only compared between maple groups. Beech and Oak controls were not included in development data. These data are summarized in Figures 2.4 and 2.5.

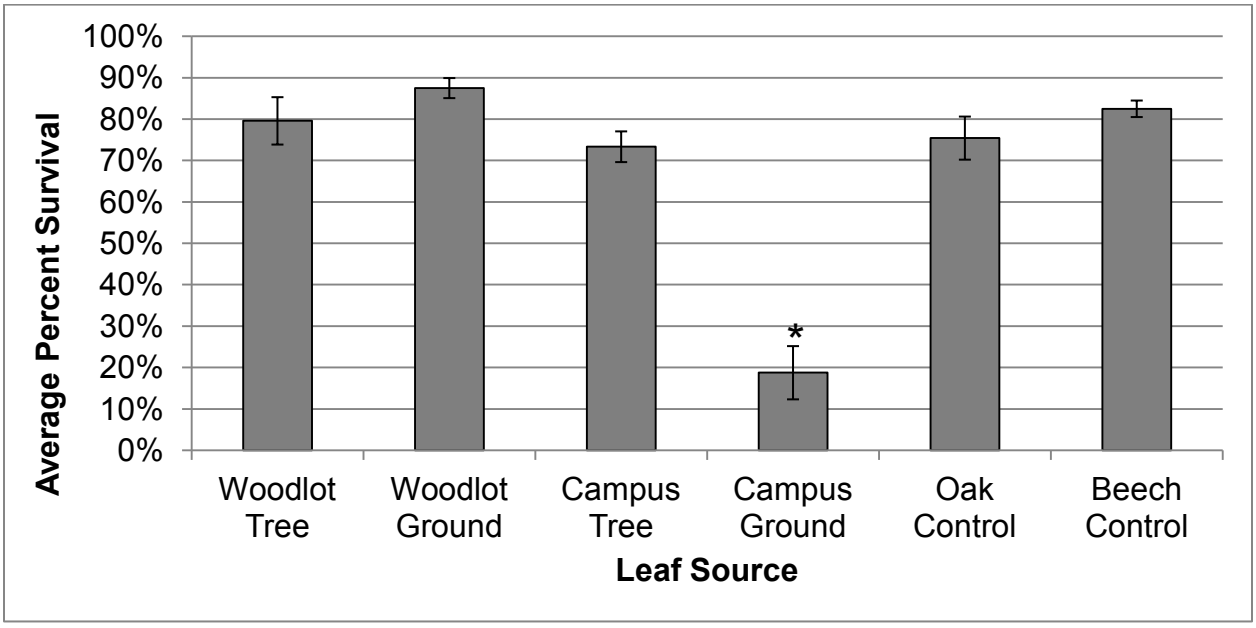


**Figure 2.4**. Average percent survival of larvae in microcosms constructed with the indicated source of leaves. Error bars indicate standard error. n=12.

**Fungal Isolation and Identification.** Two species of fungus were isolated from contaminated leaves for further experimentation and characterization. Species isolated were *Aspergillus niger* and *Fusarium oxysporum.* Both were confirmed by 18s sequencing as described above.

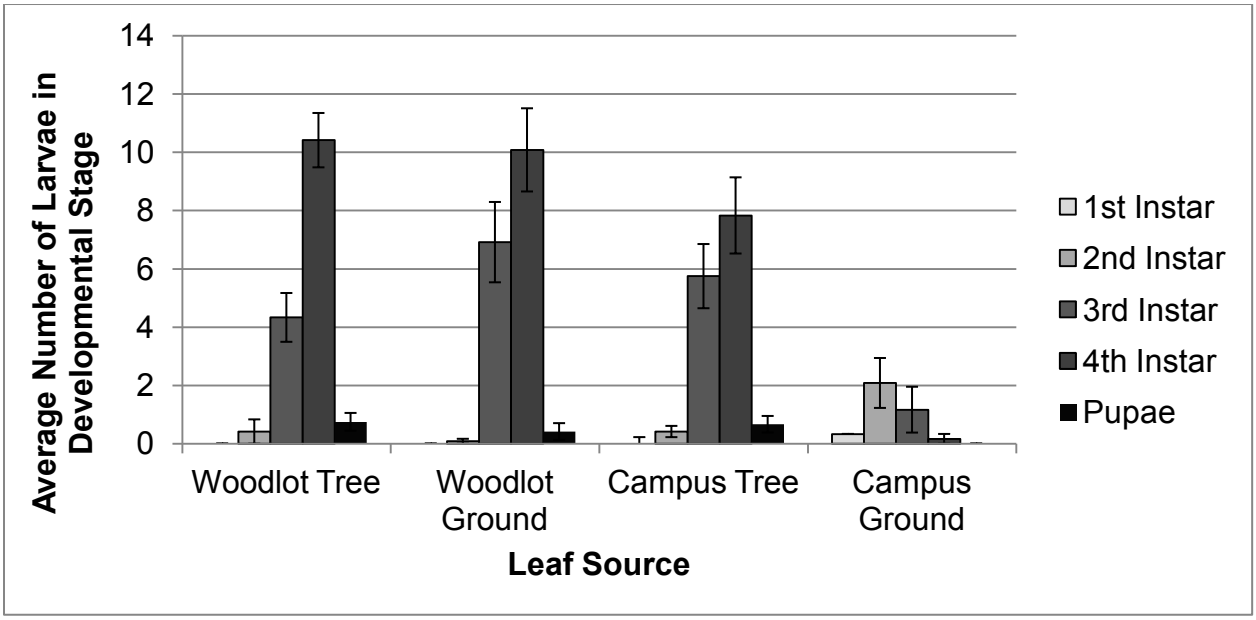


**Figure 2.5:** Average number of larvae per microcosm in the designated developmental stage. Light grey indicates early (first and second) instars, dark grey indicates late (third and fourth) instars, black indicates pupae. Error bars indicate standard error. n=12.

## Discussion

Initial observations and confirmation tests demonstrate a definite and significant effect when first instar larvae of the mosquito species *Aedes triseriatus* are grown in the presence of certain populations of leaves from the Sugar Maple, *Acer saccharum*. Most dramatically, the pronounced die-offs of larvae would suggest that there is a component

or combination of components present in the natural mosquito habitat that could be of great interest to both microbiology and entomology as a system that can be explored for potential mosquito control measures or products in the future. In fact, the presence of a copious amount of apparent fungal growth, which, if it were truly of fungal origin would be expected to produce a high level of ergosterol, demonstrating uncommonly low levels of associated ergosterol in the water column lends another interesting aspect to this exploration. The implication that perhaps it is an oomycete, and not a true fungus, that is responsible for the mortality effect is, in itself, interesting given the history of oomycetes in mosquito control in the past. In 1996, the oomycete *Lagenidium giganteum* was approved for use as a larvicide under the name Laginex in three different formulations, by the Environmental Protection Agency (USEPA 2001).It was considered established that this product was not harmful to mammals (Kerwin et al. 1990) or other off target organisms. In fact, the EPA Fact Sheet from 2001 states that

> "*Lagenidium giganteum* has no observable effects on any organisms except susceptible mosquitoes. Because the fungus is specifically used in aquatic environments, its potential harmful effects on aquatic organisms were thoroughly studied. No harmful effects were found in any aquatic or terrestrial species tested, including birds, beneficial insects, aquatic fish and invertebrates, mammals, and other animals."

However, there was some evidence to the contrary, showing that the organism *Lagenidium giganteum* may be harmful to several species of aquatic organisms, including both insects and small crustaceans (Couch 1935, Nestrud and Anderson 1994). It now appears that *L. giganteum* may also be pathogenic to mammals,

specifically, to canines (Grooters et al. 2003). Additionally, it was voluntarily removed from the market in the fall of 2011 (USEPA 2011). Given this recent development, it is of interest to look more closely at fungus and oomycetes that are pathogenic to mosquitoes, as they may provide insights into potentially mammal-pathogenic species or strains.

The isolation of *Aspergillus niger* and *Fusarium oxysporum* from the experimental microcosm is not surprising, as they are both very common species. In fact, according to the Centers for Disease Control, "Most people breathe in *Aspergillus* spores every day". However, previous research has indicated that related species can infect or kill mosquitoes of multiple genera (Hasan and Vago 1972, Teetor-Barsch and Roberts 1983, Moraes et al. 2001, Seye et al. 2009, Prakash et al. 2010), so it is of interest to see whether they are similarly effective against *Ae. triseriatus*.

# LITERATURE CITED

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**Arrivillaga, J., and R. Barrera. 2004.** Food as a limiting factor for Aedes aegypti in water-storage containers. Journal of Vector Ecology 29: 11-20.

**Borneman, J., and R. J. Hartin. 2000.** PCR primers that amplify fungal rRNA genes from environmental samples. Applied and environmental microbiology 66: 4356-4360.

**Couch, J. N. 1935.** A New Saprophytic Species of Lagenidium, with Notes on Other Forms. Mycologia 27: 376-387.

**Fish, D., and S. R. Carpenter. 1982.** Leaf Litter and Larval Mosquito Dynamics in Tree-Hole Ecosystems. Ecology 63: 283-288.

**Gaulin, E., A. Bottin, and B. Dumas. 2010.** Sterol biosynthesis in oomycete pathogens. Plant signaling & behavior 5: 258-260.

**Geraats, B. P. J., P. A. H. M. Bakker, and L. Van Loon. 2002.** Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and Arabidopsis thaliana. Molecular plant-microbe interactions 15: 1078-1085.

**Grooters, A. M., E. C. Hodgin, R. W. Bauer, C. J. Detrisac, N. R. Znajda, and R. C. Thomas. 2003.** Clinicopathologic findings associated with Lagenidium sp. infection in 6 dogs: initial description of an emerging oomycosis. Journal of veterinary internal medicine 17: 637-646.

**Hasan, S., and C. Vago. 1972.** The pathogenicity of Fusarium oxysporum to mosquito larvae. Journal of Invertebrate Pathology 20: 268-271.

**Ho, W. C., and W. H. Ko. 1997.** A simple method for obtaining single-spore isolates of fungi. Botanical Bulletin of Academia Sinica 38.

**Intermill, R. 1973.** Development of Dirofilaria immitis in Aedes triseriatus Say. Mosquito News 33: 176-181.

**Kerwin, J. L., D. A. Dritz, and R. K. Washino. 1990.** Confirmation of the safety of Lagenidium giganteum (Oomycetes: Lagenidiales) to mammals. Journal of economic entomology 83: 374-376.

**Lodge, D. J., P. J. Fisher, and B. C. Sutton. 1996.** Endophytic Fungi of Manilkara bidentata Leaves in Puerto Rico. Mycologia 88: 733-738.

**McCorkindale, N. J., S. A. Hutchinson, B. A. Pursey, W. T. Scott, and R. Wheeler. 1969.** A comparison of the types of sterol found in species of the saprolegniales and leptomitales with those found in some other phycomycetes. Phytochemistry 8: 861-867.

**Moraes, A. M. L. d., G. L. d. Costa, M. Z. d. C. Barcellos, R. L. d. Oliveira, and P. C. d. Oliveira. 2001.** The entomopathogenic potential of Aspergillus spp. in mosquitoes vectors of tropical diseases. Journal of Basic Microbiology 41: 45-49.

**Naksathit, A. T., J. D. Edman, and T. W. Scott. 1999.** Amounts of Glycogen, Lipid, and Sugar in Adult Female Aedes aegypti (Diptera: Culicidae) Fed Sucrose. Journal of Medical Entomology 36: 8-12.

**Nestrud, L. B., and R. L. Anderson. 1994.** Aquatic safety of Lagenidium giganteum: Effects on freshwater fish and invertebrates. Journal of Invertebrate Pathology 64: 228-233.

**Nnakumusana, E. 1985.** Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to Aspergillus parasiticus and its effects on fecundity and longevity of mosquitoes exposed to sporal infections in larval stages. Current Science 54: 1221.

**Onken, H., S. B. Moffett, and D. F. Moffett. 2004.** The anterior stomach of larval mosquitoes (Aedes aegypti): effects of neuropeptides on transepithelial ion transport and muscular motility. Journal of Experimental Biology 207: 3731-3739.

**Prakash, S., G. Singh, N. Soni, and S. Sharma. 2010.** Pathogenicity of Fusarium oxysporum against the larvae of Culex quinquefasciatus (Say) and Anopheles stephensi (Liston) in laboratory. Parasitology research 107: 651-655.

**Rahuman, A. A., G. Gopalakrishnan, P. Venkatesan, and K. Geetha. 2008.** Larvicidal activity of some Euphorbiaceae plant extracts against Aedes aegypti and Culex quinquefasciatus (Diptera: Culicidae). Parasitology research 102: 867-873.

**Seitz, L. M., D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard. 1979.** Ergosterol as a measure of fungal growth. *Phytopathology* 69: 1202-1203.

**Seye, F., O. Faye, M. Ndiaye, E. Njie, and J. M. Afoutou. 2009.** Pathogenicity of the fungus, Aspergillus clavatus, isolated from the locust, Oedaleus senegalensis, against larvae of the mosquitoes Aedes aegypti, Anopheles gambiae and Culex quinquefasciatus. Journal of Insect Science 9.

**Teetor-Barsch, G. H., and D. W. Roberts. 1983.** Entomogenous Fusarium species. Mycopathologia 84: 3-16.

**Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. O'guinn, T. G. Andreadis, and J. A. Blow. 2005.** An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 42: 57-62.

**USEPA. 2001.** *Lagenidium giganteum* (129084) Fact Sheet. *In* U. S. E. P. Agency [ed.].

# CHAPTER 3: QUANTIFICATION OF MORTALITY AND INHIBITION OF DEVELOPMENT OF MOSQUITO LARVAE (CULICIDAE) FOLLOWING EXPOSURE TO PURIFIED SPORE SUSPENSIONS OF *ASPERGILLUS NIGER, FUSARIUM OXYSPORUM,* AND *PYTHIUM ULTIMUM*

## Introduction

Development of larval mosquitoes has a pronounced effect on their vectorial capacity as adults. Mosquitoes that are stunted as larvae, either by starvation or competition, have been shown to be more effective at transmitting diseases such as La Crosse Encephalitis (Grimstad and Haramis 1984, Grimstad and Walker 1991). By the same token, mosquitoes that do not survive to eclosion, and thereby never develop into adults, cannot transmit disease at all. It is therefore of interest to delve further into the relationships between the larval development of disease-transmitting mosquito species and their potential pathogens.

One such disease-transmitting mosquito species is *Aedes triseriatus*, the primary vector for La Crosse Encephalitis (Sudia et al. 1971, Watts et al. 1972). This tree hole-dwelling mosquito is a daytime biter and has been known to feed on humans (Walker 1992). Another closely related species that also occasionally feeds on humans is *Aedes japonicus japonicus*, an invasive species which established populations in the United States in the late 1990's (Molaei et al. 2009). As an invasive species which shares a very similar habitat preference to that of *Ae. triseriatus*, it has been speculated that *Ae. j. japonicus* may be in competition with *Ae. triseriatus* in its natural habitat(Alto 2011). If this is the case, investigation of potential pathogens which may yield a competitive advantage to either speices are also of interest in current studies.

Following the observation of lethal and inhibitory effects when contaminated maple leaves were used to encourage microbial growth in experimental microcosms and the subsequent isolation and characterization of potential etiologic agents, it was

determined that bioassays should be run utilizing the isolated fungi and oomycetes. These tests are of value to see which, if any, of the isolated fungi are contributing most to the observed effect. Determination of the primary causative agents will allow for more in-depth examination of how they cause the observed effects. While unlikely that any of the isolated agents would be a candidate for direct use as a larvicidal agent, based on the unexpected off-target results of *Lagenidium giganteum* use (Couch 1935, Nestrud and Anderson 1994, Grooters et al. 2003), this examination may shed light on a compound or process that could be of use in the development of future mosquitocidal products.

Of additional interest is whether or not these agents act in a synergistic manner such that the combination of two or more of the isolated species provide a more robust effect than would be observed normally. There is evidence to support the idea that fungi can work in a synergistic manner when infecting host tissue. In fact, it has been shown that "parasitizing *Fusarium sp.* may weaken the insect to the extent that the latter will be predisposed to pathologies of other causes." (Teetor-Barsch and Roberts 1983). It seems logical that this would be the case with nearly any combination of pathogenic influences on an organism, as the host system would be taxed further by each new challenge and thus unable to react as strongly to any one pathogen as it might have otherwise been able to do. In order to determine whether the isolated fungi and oomycetes produced the previously observed effect either individually or in combination, a series of bioassays were run, exposing larvae of the mosquito species *Aedes triseriatus* to the isolated cultures and observing subsequent mortality and/or inhibition of development.

Another point of interest in determining the efficacy of these agents in causing mortality in mosquito larvae is investigating potential differences in efficacy based on the developmental stages of the larvae at the time of exposure. It was expected that the first instars of both *Ae. triseriatus* and *Ae. japonicus* larvae would be more susceptible to fungal pathogens, based on the susceptibility of early instars of both mosquitoes and other insects to infection by microbes and parasites (Bukhari et al. , Stairs 1965, Magnoler 1975). Based on these studies, first instars were used for the following studies unless otherwise indicated.

An additional aspect of the effects of fungal challenge on mosquito larvae that was evaluated in this study was whether there was a decrease in adult emergence after challenge. It was thought that, even if high levels of mortality were not seen when larvae were challenged with some of these fungi, there may be decreased adult emergence due to inhibited development. This would be directly relevant to mosquitoes as vectors of disease, as it is the adult stage of the mosquito that is responsible for transmission of disease in most, if not all, cases.

**Objectives and Hypotheses**. Bioassays evaluating the mortality and development of mosquito larvae exposed to fungal or oomycete pathogens were used to evaluate the following hypotheses:

**Hypothesis 1:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant mosquitocidal effects when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 2:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant inhibition of growth and development when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 3:** Mosquitocidal effects will increase with mixed-species inoculum as compared with single-species inoculum.

## Materials and Methods

**Media Preparation**. Mung bean broth was prepared according to a modification of the protocol from the University of Kentucky Department of Plant and Soil Sciences website (2006). Briefly, 1 L of milli-Q filtered water was brought to a boil in a covered 2 L Erlenmeyer flask. Once boiling, the water was removed from the heat source and allowed to sit for 30 seconds. At this point, 40 g of dried mung beans were added and the flask was swirled gently to disperse them. The beans were allowed to steep for ten minutes undisturbed. After ten minutes, the broth was poured into a clean flask or flasks, depending on the volume needed, being sure that no beans were transferred in the process. Flasks were covered with clean aluminum foil and autoclaved for 20 minutes at 123°.

3% Water Agar: Thirty grams of Bacto-agar (Becton Dickinson and Company, Franklin Lakes, NJ) were added to 1 L milli-Q filtered water and dissolved. The solution was autoclaved for 20 minutes at 123°. Plates were poured on a benchtop, using standard aseptic technique.

Czapek Yeast Agar: For Czapek Yeast Agar, a concentrate first had to be made using the following ingredients and amounts: sodium nitrate (30.0 g), potassium chloride (5.0 g), magnesium sulfate heptahydrate (5.0 g), ferrous sulfate heptahydrate (0.1 g), zinc sulfate heptahydrate (0.1 g), cupric sulfate pentahydrate (0.05 g), Milli-Q filtered water (100 mL). Components were combined and dissolved with stirring and low heat. Once Czapek concentrate was finished, Czapek Yeast Agar plates were made as follows: potassium phosphate (dibasic- 1.0 g), Czapek concentrate (10 mL), powdered yeast extract (5.0 g), sucrose (15.0 g), and agarose (15.0 g) were combined and dissolved in 1.0 L Milli-Q filtered water with stirring. Media was autoclaved for 20 minutes at 123° C. After autoclaving, media was cooled in a water bath to approximately 55° C, then Chloramphenicol and Kanamycin were added in a sterile biological safety cabinet to a final concentration of 0.05 g/L. Plates were poured on a benchtop using standard aseptic technique (Klich and voor Schimmelcultures 2002).

CMA-PARP: For Corn Meal Agar (CMA) plates, 17 g of Corn Meal Agar was dissolved in Milli-Q filtered water in an Erlenmeyer flask and autoclaved for 20 minutes at 123° C. The media was allowed to cool to approximately 55° C, then the following antibiotics were added with gentle swirling to distribute: Ampicillin (0.25 g dissolved in 50% ethanol), Rifampicin (0.01 g dissolved in DMSO), Pimaricin (25 mg dissolved in Milli-Q water), Penta-Chloro-Nitro-Benzene (PCNB, 0.025 g dissolved in warn 95% ethanol). Plates were poured on a benchtop using standard aseptic technique (Vitoreli).

cV8A: Clarified v8 juice was made as follows: Calcium carbonate was added to room temperature V8 juice (CSC Brands LP, 2 g per 200 mL). The mixture was centrifuged for 20 minutes at 4000 rpm to clarify. To make 3% clarified V8 Agar (cV8A),

30 mL of clarified V8 juice and 15 g of Bacto-agar were added to 1 L of Milli-Q filtered water and autoclaved for 20 minutes at 123° C. The media was cooled to approximately 55° C and plates were poured on the benchtop using standard aseptic technique (Jeffers 2006).

**Fungal Spore Suspension Preparation.** Fungal spore suspensions were produced as follows: Isolated *Fusarium* cultures were grown on 3% water agar for five to seven days at room temperature, until plates reached nearly full coverage. At this point, plates were divided into approximately sixteen pieces each and transferred into prepared mung bean broth- one 100 mm plate per liter of broth. Inoculated broth was allowed to sit on a benchtop at room temperature for approximately two weeks, with occasional swirling of the flask. After two weeks of growth, the broth was filtered through sterile gauze to remove hyphae and transferred to 50 mL centrifuge tubes. Spore solutions were centrifuged for 15 minutes at 4000 rpm and the pellets were collected and combined into one vessel. The concentration of spores in this solution was determined using an improved Neubauer phase contrast hemocytometer (Hausser Scientific, Horsham, PA). Concentrations were calculated as the mean of ten to twelve counts. Spore suspensions were then diluted to a standard concentration using mung bean broth.

*Aspergillus* cultures were grown on CYA slants in sterile 50 mL centrifuge tubes with polypropylene screw-on caps at room temperature for approximately one week. After sufficient growth was observed, 15 mL sterile Milli-Q filtered water was added to each tube and tubes were capped tightly and shaken vigorously and/or vortexed to suspend the hydrophobic spores in the water (Brown and Salvo 1992). Spore

concentrations were determined using an improved Neubauer phase contrast hemocytometer (Hausser Scientific, Horsham, PA). Spore suspensions were then diluted to a standard concentration using sterile water.

*Pythium* cultures were grown continually on CMA-PARP. A 5 mm x 5 mm piece of actively growing culture was transferred to the center of a 3% cV8A plate and allowed to grow for one week in the dark at room temperature. After one week, this plate was flooded with 15 mL sterile water and allowed to sit for one to two hours (Pettitt et al. 2002). The water was then pipetted off the surface of the plate and collected in a centrifuge tube. Spore concentrations were not able to be accurately measured by the researcher, so a standardized amount of inoculum was used in laboratory microcosms, rather than a final spore concentration. It was estimated that spore concentrations would be relatively high (similar to those seen in *Aspergillus* solutions), but this could not be confirmed.

**Single Species Laboratory Microcosms.** Laboratory microcosm bioassays were run with single fungal species to establish the efficacy of each species in killing first instar larvae of the mosquito species being studied, with the emphasis on *Ae. triseriatus*, though *Ae. japonicus* was also challenged with *Aspergillus*. Different dosages of each fungal species were also evaluated.

*Aspergillus/Ae. triseriatus*: *Aspergillus* spores were prepared as discussed above. Microcosms were constructed in 16 ounce food storage containers, as discussed in the previous chapter and contained: 200 mL Milli-Q filtered water, 2 mL treehole inoculum, 1 g dried ground-collected beech leaves, 20 first instar *Ae. triseriatus* larvae. *Aspergillus* spores were added to reach the following final concentrations:

Control= no inoculum, Low Dose= 1.45 x $10^5$ spores/mL, Medium Dose= 1.45 x $10^6$ spores/mL, High Dose= 2.9 x $10^6$ spores/mL. Microcosms were run for one week at room temperature. After one week, larvae were checked for survival, counted, and preserved in 70% ethanol.

*Aspergillus/Ae. japonicus*. Bioassays evaluating the survival of *Ae. japonicus* exposed to *Aspergillus* spores were run as above, with all concentrations the same. These experiments were not, however, run concurrently, so data from the two trials could not be compared directly during statistical analysis.

*Fusarium*/*Ae. triseriatus*. Bioassays evaluating the survival of *Ae. triseriatus* exposed to *Fusarium* spores were run as above, with final concentrations as follows: Control= no inoculum, Low Dose= 2.5 x $10^3$ spores/mL, Medium Dose= 2.5 x $10^4$ spores/mL, High Dose= 2.5 x $10^5$ spores/mL.

**Mixed Species Laboratory Microcosms.** Two laboratory microcosm trials were run in preparation for simulated field microcosms. Initial mixed species microcosms were run using a modification of the 16 ounce microcosm system described in the previous chapter. Microcosms contained 200 mL Milli-Q filtered water, 1 g dried ground-collected beech leaves, 2 mL treehole inoculum, and spore solutions to reach the following final concentrations: *Aspergillus*- 1.45 x $10^6$ spores/mL, *Fusarium*- 25,000 spores/mL. Microcosms were set up and twenty first instar larvae were added immediately. For Trial 1, all larvae were *Ae. triseriatus.* In Trial 2, ten *Ae. triseriatus*

larvae and ten *Ae. japonicus* larvae were added to each microcosm. Microcosms were run for one week in an incubator set to 28° C, then larvae were removed, counted, and preserved in 70% ethanol for further analysis. Water samples (50 mL each) were taken for DNA extraction. Statistical analysis was via two-factor analysis of variance (two-way ANOVA) and Tukey's Honest Significant Difference.

**Mixed Species Simulated Field Microcosms.** Due to the fact that laboratory conditions are typically severely limited when compared with the immense complexity of natural systems, simulated field microcosms were created using standard used automotive tires. These microcosms were designed to more closely represent the conditions found in the natural habitats of these tire-dwelling mosquitoes. Elements that contribute to the natural simulation of these microcosms include mixed species locally-collected leaf inputs, tire-based systems, and placement in a beech-maple woodlot in which many native populations of *Aedes triseriatus* are found.

Tire preparation was as follows: tires were screened off using 1 x 1.5 mm aluminum window screening and silicone caulk to create an enclosed space in the base of the tire. This enclosure was intended to exclude wild mosquitoes and other insects that might otherwise oviposit in the experimental area and to prevent the escape of any experimental mosquitoes that might eclose prior to the end of the trial.

Trial 1: Microcosms contained: 1 L Milli-Q filtered water, 5 g dried leaf litter (mixed species collected from the floor of the woodlot in which tires were stationed). After one week, on Day 1, 100 first instar *Ae. triseriatus* larvae and 100 first instar *Ae. japonicus* larvae were added for a total of 200 larvae per tire. Larvae were allowed to

acclimate in the tire microcosms for 24 hours prior to the addition of fungal inocula. Fungal inocula were added to experimental containers on Day 2. Fungal inocula were added to the following final concentrations: *Aspergillus*: 1.45 x $10^6$ spores/mL, *Fusarium*: 25,000 spores/mL. In Trial 1, treatment tires received fungal spores and controls were untreated Microcosms were allowed to run for one week. After one week, the entire contents of the tire microcosms were removed and transferred to the laboratory where they were processed.

Trial 1 Processing: Tire contents were transferred to ceramic-coated larval rearing trays. Leaves were removed and stored at -80° C. Water samples (50 mL each) were taken for DNA extraction. Larvae were checked for survival, counted, and incubated at 28° C for 48 hours, then recounted and preserved in 70% ethanol.

Trial 2: Microcosms contained: 1 L Milli-Q filtered water, 5 g dried leaf litter (mixed species collected from the floor of the woodlot in which tires were stationed). For this trial, poor larval hatch led to a necessary staggering of the addition of larvae to experimental containers. After one week, on Day 1, 50 first instar *Ae. triseriatus* larvae and 50 first instar *Ae. japonicus* larvae were added for a total of 100 larvae per tire. The remaining 100 larvae, 50 *Ae. triseriatus* and *Ae. japonicus* larvae, were added the following day for a final total of 200 larvae per tire. Larvae were allowed to acclimate in the tire microcosms for 24 hours after the second set of larvae were added, prior to the addition of fungal inocula. Fungal inocula were added on Day 3. Fungal inocula were added to the following final concentrations: *Aspergillus*: 1.45 x $10^6$ spores/mL, *Fusarium*: 25,000 spores/mL. In Trial 2, control tires received autoclaved fungal inocula,

to provide a more appropriate negative control. Autoclaved fungal spores were also tested for viability by plating on appropriate media, as discussed in the fungal growth section. After one week, the entire contents of the tire microcosms were removed and transferred to the laboratory where they were processed.

Trial 2 Processing: Tire contents were transferred to cold storage (4° C) overnight. Then leaves were removed and stored at -80° C, water samples were taken for DNA extraction, and larvae were counted and preserved in 70% ethanol.

Preserved larvae from both trials were examined under a dissecting microscope to determine developmental stage and look for any visible signs of infection. Statistical analysis was via two-factor analysis of variance (two-way ANOVA) and Tukey's Honest Significant Difference.

**Single Species Laboratory Nanocosms**. Single species laboratory nanocosms were run with single larvae in the wells of a standard 24 well tissue culture plate (Costar, Corning Incorporated, Tewksbury, MA). Plates were set up as follows: 0.2 mg Tetramin brand fish food (Tetra Werke, Melle, Germany) and approximately 2.5 mL of water were added to each well (water volumes were adjusted to account for the volume of the fungal inoculum, for a final volume of 2.5 mL). Once plates were set up, larvae were added. For this experiment, larvae at four different developmental stages were used. Larval instars were estimated based on developmental time (ie- Larvae less than 24 hours old were considered first instars, larvae approximately three days old were considered second instars, and larvae five days old were considered third instars). Populations were checked to ensure that the majority of the larvae were of appropriate

size and development to meet the indicated developmental stage, but each individual larva was not checked prior to use. Larvae were divided into 32 treatment groups, as outlined in Table 3.1, with 24 larvae per group. After all larvae were in plates, fungal inoculum was added to appropriate wells as follows: *Aspergillus/Fusarium*- Fungal inoculum was added to reach a final concentration of $3.0 \times 10^6$ spores/mL, *Pythium*-0.095 mL were added to each well. Nanocosms were allowed to run for one week with regular feeding (approximately 0.1 mg /larva/day). At the end of one week, larvae were checked for survival, counted, and preserved in 70% ethanol.

**Multi Species Laboratory Nanocosms**. Multi-species laboratory microcosms were set up as with the single species nanocosms described above, but only *Ae. triseriatus* larvae were used. Treatment groups for this experiment were: Untreated controls, *Aspergillus*, *Fusarium, Pythium, Aspergillus/Fusarium, Aspergillus/Pythium, Fusarium/Pythium, Aspergillus/Fusarium/Pythium*, where names in the format X/Y indicate that multiple fungal species were used in combination in those wells. There were 24 mosquitoes per treatment group. Final fungal concentrations were $1.45 \times 10^7$ spores/mL where accurate counts could be obtained. For *Aspergillus/Fusarium* treatment group wells, inocula were standardized to an equal concentration and the same amount was added of both, to reach the desired final total concentration. For *Pythium*-treated wells, consistent amounts of *Aspergillus* and *Fusarium* were used and a volume of *Pythium* consistent with that added for the other two species was used. The experiment was allowed to run for one week, then mortality was recorded and larvae were preserved in 10% neutral buffered formalin.

**Table 3.1.** Distribution of treatment groups for Single Species Laboratory Microcosms experiment.

| Group | Mosquito Species | Fungal Inoculum | Instar | n |
|---|---|---|---|---|
| 1 | *Ae. triseriatus* | *Aspergillus* | 1 | 24 |
| 2 | *Ae. triseriatus* | *Aspergillus* | 2 | 24 |
| 3 | *Ae. triseriatus* | *Aspergillus* | 3 | 24 |
| 4 | *Ae. triseriatus* | *Aspergillus* | 4 | 24 |
| 5 | *Ae. triseriatus* | *Fusarium* | 1 | 24 |
| 6 | *Ae. triseriatus* | *Fusarium* | 2 | 24 |
| 7 | *Ae. triseriatus* | *Fusarium* | 3 | 24 |
| 8 | *Ae. triseriatus* | *Fusarium* | 4 | 24 |
| 9 | *Ae. triseriatus* | *Pythium* | 1 | 24 |
| 10 | *Ae. triseriatus* | *Pythium* | 2 | 24 |
| 11 | *Ae. triseriatus* | *Pythium* | 3 | 24 |
| 12 | *Ae. triseriatus* | *Pythium* | 4 | 24 |
| 13 | *Ae. triseriatus* | Control | 1 | 24 |
| 14 | *Ae. triseriatus* | Control | 2 | 24 |
| 15 | *Ae. triseriatus* | Control | 3 | 24 |
| 16 | *Ae. triseriatus* | Control | 4 | 24 |
| 17 | *Ae. japonicus* | *Aspergillus* | 1 | 24 |
| 18 | *Ae. japonicus* | *Aspergillus* | 2 | 24 |
| 19 | *Ae. japonicus* | *Aspergillus* | 3 | 24 |
| 20 | *Ae. japonicus* | *Aspergillus* | 4 | 24 |
| 21 | *Ae. japonicus* | *Fusarium* | 1 | 24 |
| 22 | *Ae. japonicus* | *Fusarium* | 2 | 24 |
| 23 | *Ae. japonicus* | *Fusarium* | 3 | 24 |
| 24 | *Ae. japonicus* | *Fusarium* | 4 | 24 |
| 25 | *Ae. japonicus* | *Pythium* | 1 | 24 |
| 26 | *Ae. japonicus* | *Pythium* | 2 | 24 |
| 27 | *Ae. japonicus* | *Pythium* | 3 | 24 |
| 28 | *Ae. japonicus* | *Pythium* | 4 | 24 |
| 29 | *Ae. japonicus* | Control | 1 | 24 |
| 30 | *Ae. japonicus* | Control | 2 | 24 |
| 31 | *Ae. japonicus* | Control | 3 | 24 |
| 32 | *Ae. japonicus* | Control | 4 | 24 |

**Adult Emergence Nanocosms.** Single larva nanocosms were also utilized to analyze adult emergence after exposure to fungal challenge. Fungal treatments were with *Aspergillus* and *Fusarium* spores. There were 24 larvae per treatment group. Nanocosms were set up in 24 well tissue culture plates (Costar, Corning Incorporated, Tewksbury, MA) as follows: 2.325 mL Milli-Q filtered water, 0.075 mL fungal inoculum to reach a final concentration of $1.45 \times 10^6$ spores/mL, and 0.2 mg Tetramin brand fish food (Tetra Werke, Melle, Germany). One first instar larva was added to each well. Plates were checked every day and pupae were removed to eclosion containers consisting of a 1 ounce cup of Milli-Q filtered water inside a ventilated 16 ounce food storage container, where they remained until they eclosed. Time to pupation and time to eclosion were both recorded.

***Anopheles* nanocosms.** Based on findings in a multi-species pathology assay (unpublished results), it was determined that an addition bioassay should be run in which larvae of the mosquito species *Anopheles gambiae* (Kisumu strain), one of the primary vectors for human malaria in Africa, were challenged with *Fusarium* spores. Nanocosms were set up as above. Briefly, 2.325 mL Milli-Q filtered water, 0.075 mL fungal inoculum to reach a final concentration of $1.45 \times 10^6$ spores/mL, and 100 µL of a 10 mg/mL solution of active dry yeast in water were combined in each well of 24 well tissue culture plates (Costar, Corning Incorporated, Tewksbury, MA). One larva (approximately 48 hours old) was added to each well, with a total of 48 larvae per treatment group (*Fusarium*-treated and untreated control). Larvae were fed another 100 µL of active dry yeast solution on day 4 of the experiment. On day 7, mortality was

recorded and larvae were preserved in 10% neutral buffered formalin. Body length of preserved larvae was recorded as an indicator of development.

## Results

**Single Species Laboratory Microcosms.** *Aspergillus/Ae. triseriatus:* In the *Aspergillus/Ae. triseriatus* microcosm bioassay, a dose-response pattern was evident based on ANOVA ($p < 0.001$)and pairwise t-tests with Bonferroni's adjustment. Only the high dose treatment group showed increased mortality when compared with the untreated control ($p < 0.001$). The mortality in the high dose group was also significantly higher than in the low and medium dose treatment groups ($p < 0.001$). The average survival was 77% for controls, 68% for the low dose treatment group, 69% for the medium dose treatment group, and 20% for the high dose treatment group (Figure 3.1).

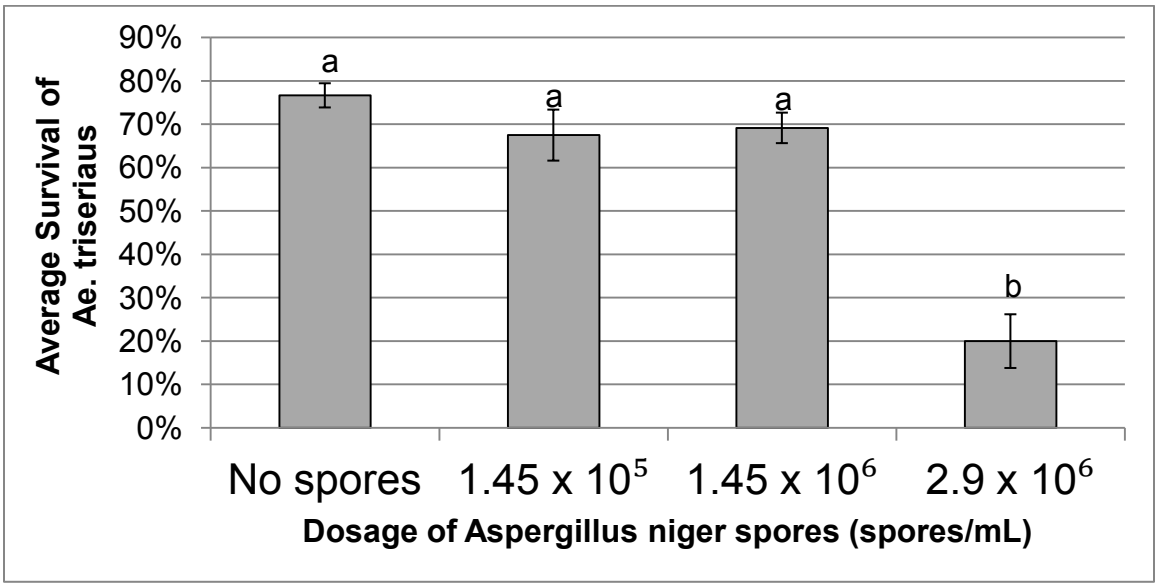


**Figure 3.1.** Average survival of *Ae. triseriatus* larvae after exposure to various doses of spores of the fungus *Aspergillus niger.* Error bars indicate standard error. n=6.

*Aspergillus/Ae. j. japonicus*. In the *Aspergillus/Ae. j japonicus* microcosm bioassay, a robust dose-response pattern was observed (Figure 3.2). The non-parametric Kruskal-Wallis test was used for initial analysis of the data to accommodate the zero variance present in the high dose group. This test indicated that there was at least one significant difference present ($X^2$ =20.5, df=3, p<0.005). Further testing using Bonferroni-corrected t-tests indicated that there were significant differences between all groups except the control and low doses (p<0.005 in all cases). That is, the medium and high dose treatment groups both showed increased mortality when compared to the control and low dose groups, and the high dose treatment group showed increased mortality when compared to the medium dose group. The average survival was 99% for both control and low dose treatment groups, 33% for the medium dose treatment group, and 0% for the high dose treatment group.

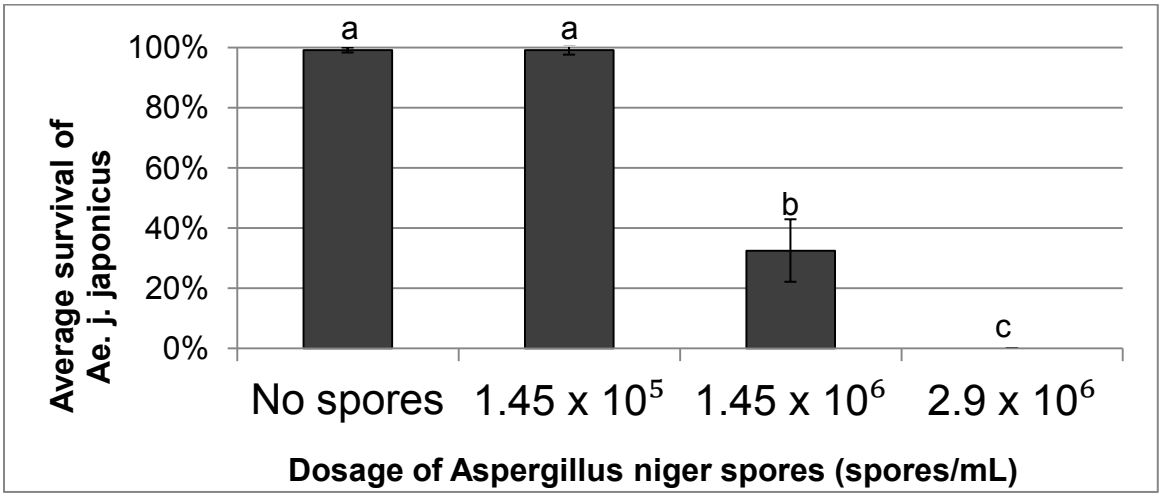


**Figure 3.2.** Average survival of *Ae. j. japonicus* larvae after exposure to various doses of spores of the fungus *Aspergillus niger.* Error bars indicate standard error. n= 6.

*Fusarium/Ae. triseriatus*. In the bioassay examining the effect of challenge with *Fusarium* spores on *Ae. triseriatus*, Kruskal-Wallis' test showed no significant relationship between dosage and mortality ($X^2$ =6.85, df=3, p=0.0767, Figure 3.3).

**Mixed Species Laboratory Microcosms.** In Trial 1, survival was 83% in controls and 8.3% in treatment containers (Figure 3.4a, t-test, df=8.45, p< 0.001). In Trial 2, a two-way ANOVA showed that significant results were obtained based on mosquito species (p<0.005), treatment group (p<0.005), and the interaction between those two factors (p<0.05). *Ae. triseriatus* survival was 82% in controls and 13% in treatment containers (Figure 3.4b. Tukeys Honest Significant Difference, p<0.005). *Ae. japonicus* survival was 87% in controls and 63% in treatment containers, which was not significantly different (Figure 3.4b. Tukey's Honest Significant Difference, p=0.217). In both treatments (p<0.005) and controls (p<0.005), *Ae. j. japonicus* survival was significantly higher than that of *Ae. triseriatus*.

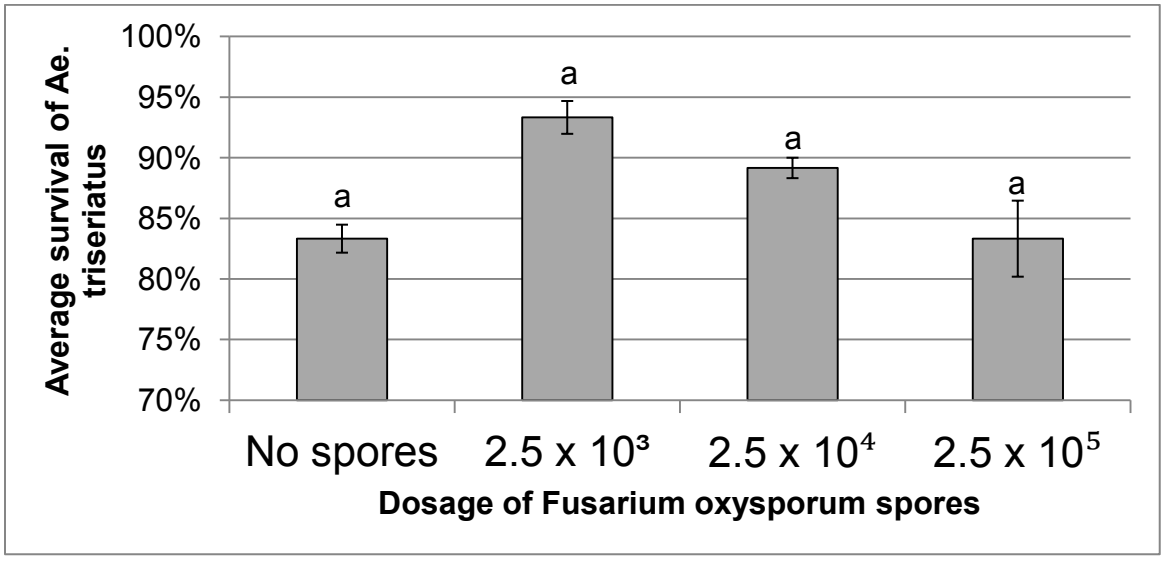


**Figure 3.3.** Average survival of *Ae. triseriatus* larvae after exposure to various doses of spores of the fungus *Fusarium oxysporum.* Error bars indicate standard error. n= 6.

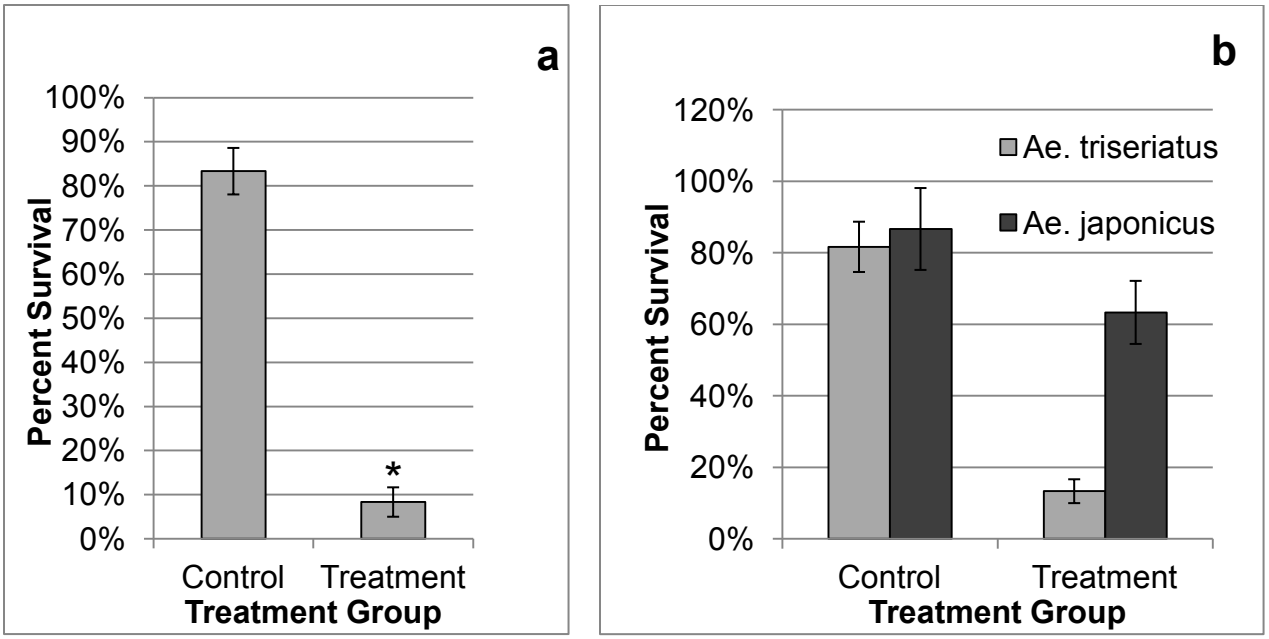


**Figure 3.4.** Average survival of *Aedes* larvae after challenge with mixed species (*Aspergillus* and *Fusarium*) fungal inoculum in laboratory microcosms. Error bars indicate standard error. a) *Aedes triseriatus* survival b) *Ae. triseriatus and Ae. j. japonicus* survival. n=6. *p<0.005

**Mixed Species Simulated Field Microcosms.** In Trial 1, two-way ANOVA showed that there was a significant effect based on mosquito species ($p<0.005$), but not based on treatment group ($p=0.572$) or interaction of these two factors ($p=0.858$). Based on the results of a Tukey's Honest Significant Difference analysis, *Ae. japonicus* demonstrated significantly higher survival in treatment containers ($p<0.05$). Survival was not significantly different between treatment and control microcosms for either species ($p> 0.05$). In Trial 2, there was no significance found between any of the experimental groups in a two-way ANOVA ($p>0.05$). Survival was not significantly different between treatment and control microcosms for either species ($p> 0.05$, Figure 3.5b). No visible signs of infection were noted in larvae from either Trial.

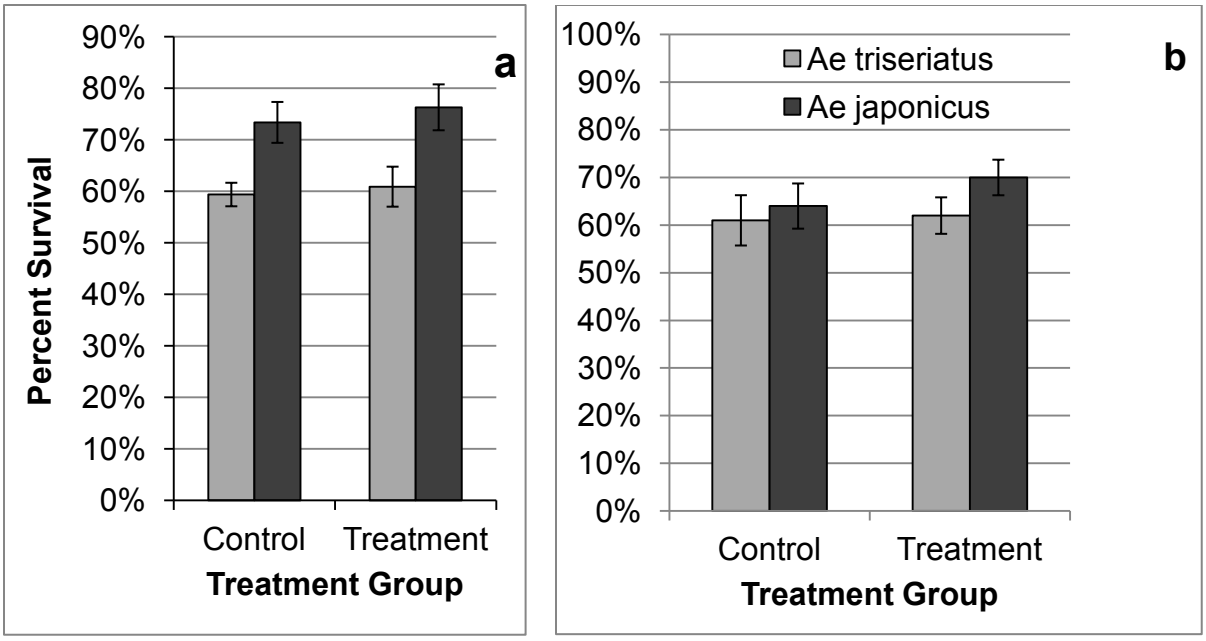


**Figure 3.5.** Average survival of *Aedes* larvae after challenge with mixed species (*Aspergillus* and *Fusarium*) fungal inoculum in simulated field microcosms. Error bars indicate standard error. a) Trial 1 b) Trial 2. Trial 1 n= 8, Trial 2 n=9.

**Single Species Laboratory Nanocosms**. When *Ae. triseriatus* larvae were challenged with *Aspergillus* spores, a clear correlation was evident between age/development of the larvae and survival. In nanocosms containing first instar larvae, there were no survivors. Survival was 8%, 33%, and 75% for second, third, and fourth instars, respectively. As a result of the binomial nature of the data (alive vs dead), pairwise tests of proportion were run and showed significant differences between treatment groups of different developmental stages within the *Ae. triseriatus*/*Aspergillus* family, as indicated in Figure 3.6. No other significant differences were seen within the *Ae. triseriatus* treatment groups. In the *Ae. japonicus/Aspergillus* treatment groups, the first instar larvae showed significantly lower survival than any of the other groups ($p < 0.01$), and the second instar survival was significantly lower than that of third instars

(p= 0.042). No other significant differences were seen within *Ae. japonicus* treatment groups (Figure 3.7). Due to the fact that not all larvae were examined prior to use, It is possible that not all larvae were within the indicated developmental stage at the time the experiment was run, however, this does not seem likely to have caused a significant change in the outcome of the experiment, and cursory examination showed that the vast majority of the larvae were of the appropriate developmental stage.

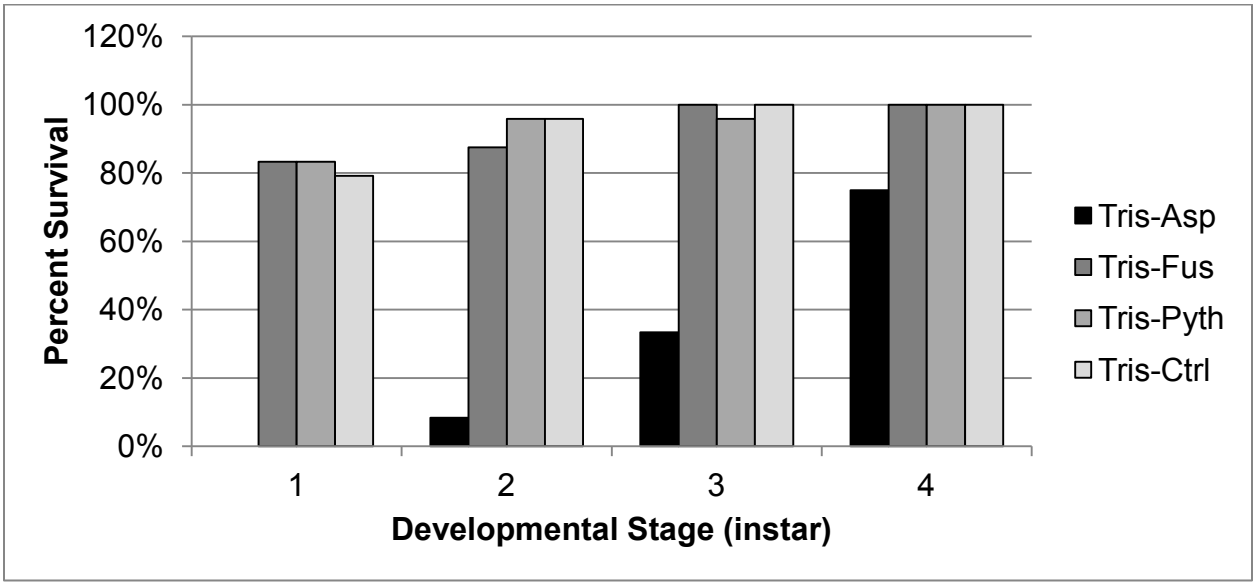


**Figure 3.6.** *Ae. triseriatus* survival by treatment group. Note the correlation between survival and age of the larva at the beginning of the experiment in *Aspergillus* groups. (Tris= *Aedes triseriatus*, Asp= *Aspergillus*, Fus= *Fusarium*, Ctrl= Untreated Control.) n=24.

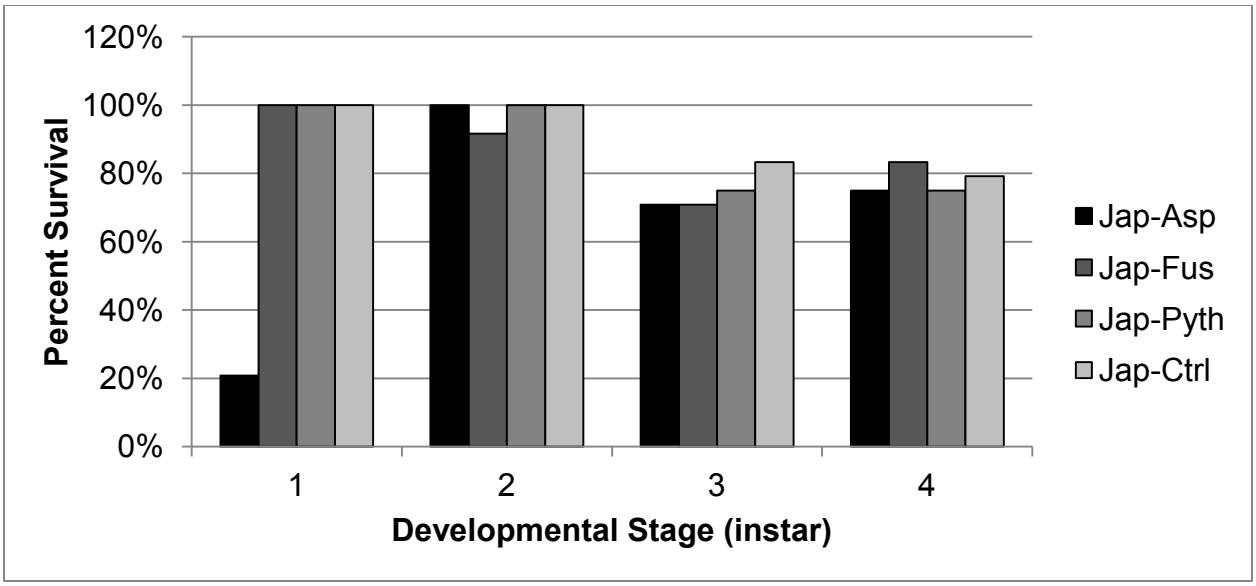


**Figure 3.7.** *Ae. j. japonicus* survival by treatment group. Note the low survival of first instar larvae challenged with *Aspergillus*. (Jap= *Aedes japonicus japonicus*, Asp= *Aspergillus*, Fus= *Fusarium*, Ctrl= Untreated Control.) n=24.

**Multi Species Laboratory Nanocosms**. *Aspergillus*-treated nanocosms appeared to show decreased survival when compared with other treatment groups, although in a pairwise test of proportion, this relationship did not reach statistical significance. Survival is shown in Figure 3.8.

**Adult Emergence Nanocosms.** *Aspergillus*-treated larvae showed significantly decreased total emergence when compared to *Fusarium*-treated and untreated control larvae in pairwise tests of proportion ($p < 0.05$). *Fusarium*-treated larvae did not show a difference in survival when compared with untreated controls. Cumulative emergence patterns are shown in Figure 3.9.

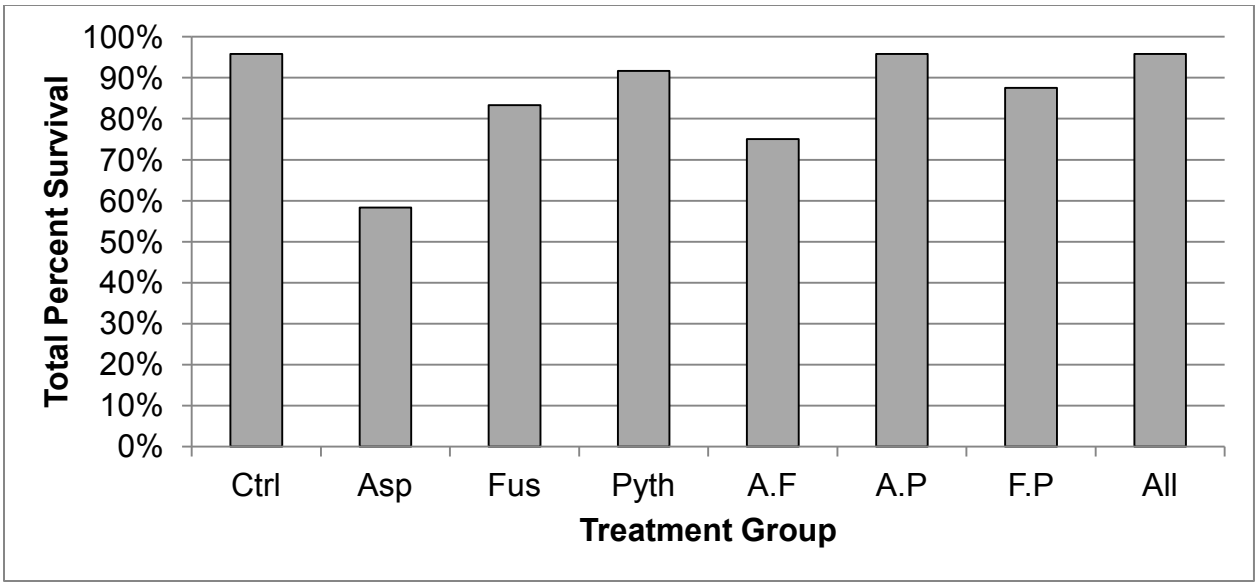


**Figure 3.8.** Survival of *Ae. triseriatus* larvae when challenged with single- and mixed-species fungal inocula. Ctrl: Control, Asp: *Aspergillus*, Fus: *Fusarium*, Pyth: *Pythium*, A.F: *Aspergillus/Fusarium*, A.P: *Aspergillus/Pythium*, F.P: *Fusarium/Pythium*, All: All three fungal species. n=24.

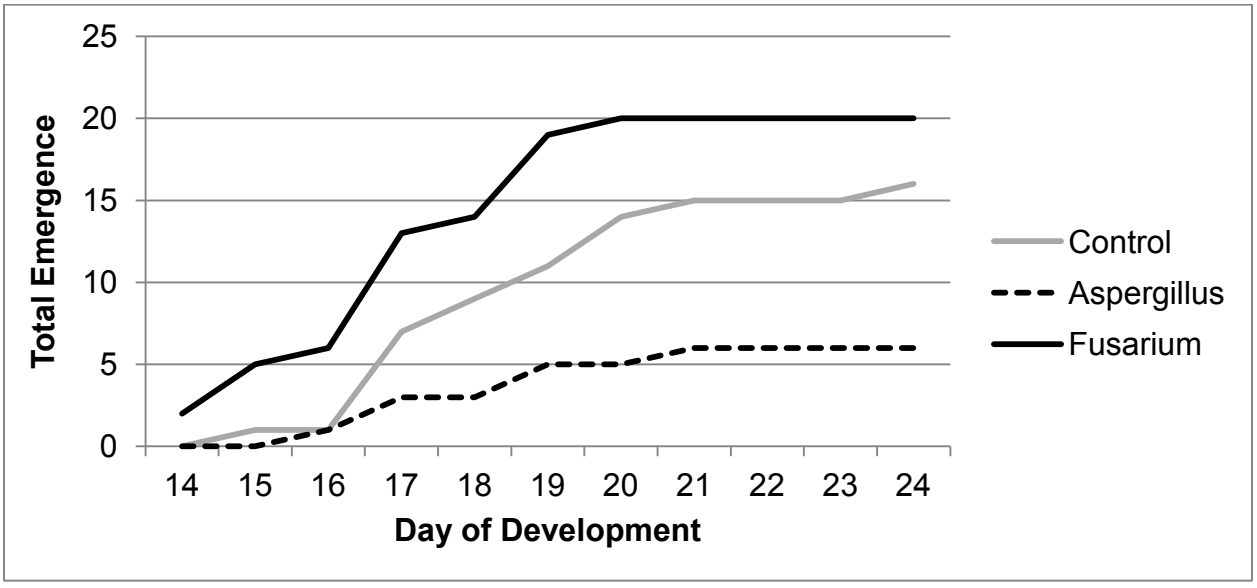


**Figure 3.9.** Cumulative adult emergence of *Ae. triseriatus* challenged with *Aspergillus* or *Fusarium* spores as larvae. n=24.

***Anopheles* nanocosms.** There was a clear and significant difference in survival between the untreated control *Anopheles gambiae* larvae and the *Fusarium*-treated larvae (pairwise test of proportion, $p<0.001$). Survival was 65% for control larvae and 12.5% for treated larvae (Figure 3.10). In addition to showing much higher mortality, the treatment group larvae were considerably smaller than their untreated counterparts when body lengths were measured.

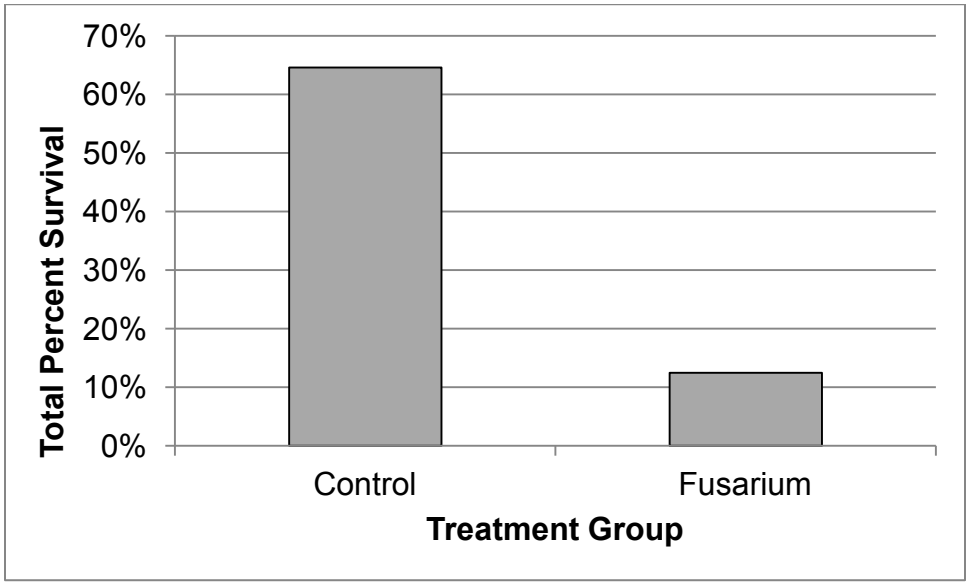


**Figure 3.10.** Survival of *Anopheles gambiae* larvae challenged with spores of the fungus *Fusarium oxysporum.* n=48.

## Discussion

Overall, the bulk of the hypotheses for these studies were unsupported by the findings of the experiments. *Aspergillus* was the only tested isolate that showed repeated significant lethal or developmental effects against larvae of *Aedes* mosquitoes. In addition, despite the lack of a significant effect, the lowest survival in the mixed

species microcosm assay was observed in the groups exposed to strictly *Aspergillus* spores, rather than a combination of species, as predicted.

**Single Species Laboratory Microcosms.** There were significant dose-response effects apparent for both *Ae. triseriatus* and *Ae. japonicus* after exposure to *Aspergillus* spores. Interestingly, the relationship between the survival rates of the two mosquito species are inverted from what would be expected based on the results of the mixed species microcosms, both in the laboratory and in the field, where *Ae. japonicus* shows increased survival when compared with *Ae. triseriatus.* However, the larvae in the mixed species microcosms were in the same experimental containers, and thus conditions were all identical. This was not the case with the single species microcosms, which were run at two separate times, rather than concurrently. As such, it is possible that, despite efforts to control all variables, something was different between the two setups that would account for the different results, and no direct comparison between the two experiments can reasonably be made. For example, there may have been something that caused a decrease in viability of the *Aspergillus* spores used for the *Ae. triseriatus* bioassay, which could easily have been missed, as there were no checks for viability during all experiments. This is something that should be added in future experiments of this variety to avoid such possible interfering factors.

The lack of a significant mortality effect in the *Fusarium* bioassay is not terribly surprising, as relatively low spore concentrations were used. These numbers were originally based on the amounts of spores that could be generated using the prepared materials and were maintained after preliminary trials (unpublished data) with mixed *Aspergillus/Fusarium* inoculum showed a robust, observable mortality effect.

**Mixed Species Laboratory Microcosms**. The results of Trial 2 show a very clear difference in survival between the two species in a competitive situation, with *Ae. japonicus* showing significantly higher survival. This is very interesting, as little is currently known about what competitive advantages *Ae. japonicus* might have that would allow it to out-compete *Ae. triseriatus* in the natural treehole environment. (Bevins 2007, Andreadis and Wolfe 2010). This provides a potential starting point for future research into the competitive qualities of these two species as they reflect the ability of the mosquitoes to survive fungal and bacterial challenges in these microbe-rich environments.

**Mixed Species Simulated Field Microcosms.** Again in this set of trials, *Ae. japonicus* demonstrates an apparent increased ability to survive a challenge with common fungal spores when compared with *Ae. triseriatus*. This effect was not as universal in the second trial as it appears to have been in the first trial or the laboratory microcosms. However, this repeated observation of differential survival does contribute to the potential for interesting future work investigating the competitive difference of these two species relating to microbial challenge.

**Single Species Laboratory Nanocosms.** Based on the known susceptibility of early instars of other insect species to infectious agents (Stairs 1965, Magnoler 1975), as well as prior studies that had shown increased susceptibility of early instars to fungal infection (Hasan and Vago 1972), it was expected that first instar larvae would show the highest levels of mortality of the tested developmental stages. This was the case in those *Ae. triseriatus* groups exposed to *Aspergillus* spores. A similar survival pattern was suggested in the other *Ae. triseriatus* groups, but was not robust enough to reach

statistical significance (Figure 3.6). This hypothesis was also supported with *Ae. japonicus* larvae exposed to *Aspergillus* spores. However, contrary to expectations, the overall survival of third and fourth instar larvae of *Ae. japonicus* was lower than that of second and, in most cases, first instar larvae, though this relationship did not reach statistical significance. Of the different fungi species tested, only *Aspergillus* showed significant levels of mortality in any of the treatment groups. The lack of effect with *Pythium* treatment groups may be due, in part, to the fact that zoospores were not able to be quantified, and as a result, may have been present only in low quantities or absent entirely. In future work, protocols should be improved to ensure the presence and viability of accurately quantifiable *Pythium* zoospores in the experimental microcosms.

**Multi Species Laboratory Nanocosms.** No significant results were evident in this experiment, though it was suggested that *Aspergillus* showed decreased survival and, in fact, if statistical analysis is run for individual groups, rather than pairwise comparisons across the board, there does appear to be a significant difference between the *Aspergillus*-treated group and untreated controls. The lack of significant results in this experiment is likely due to insufficient sample size given the number of treatment groups or decreased efficacy of spore solutions as compared to previous experiments.

**Adult Emergence Nanocosms.** *Aspergillus* spores showed a significant decrease in adult emergence when compared with untreated controls and *Fusarium*-treated microcosms. This is directly relevant to both increasing the knowledge base regarding mosquito development in nature, where they may be encountering these fungi, and to the capacity of these species for disease transmission. As it is the adult

stage of the *Aedes* mosquitoes that transmits disease, if fewer mosquitoes are reaching adulthood, this has a direct effect on potential disease transmission.

***Anopheles* nanocosms.** The ability of any agent to cause mortality or inhibition of development in the malaria vector *Anopheles gambiae* is of significant interest to the field of malariology. To date, it appears that little to no research has been done on the effects of *Fusarium oxysporum* infection on *Anopheles gambiae*. Thus, while the current study evaluated this only at a very preliminary level, it provides an intriguing avenue for further research into the relationship and interactions existing between these two species. For instance, it was observed during the course of the experiment that some of the larvae appeared tangled in fungal growth, but as observations were not made of each larva each day, it is impossible to know whether this entanglement was the cause of death or occurred posthumously. Regarding the difference in body length, all measurements were made after the experiment was concluded and mortality was not recorded prior to day 7, so it is probable that some or all of the difference in body length is due to the treatment group larvae dying toward the beginning of the experiment, before they were able to reach the later developmental stages that the surviving larvae did. Future experiments should involve a more direct comparison of daily growth, perhaps via destructive sampling to check for average daily growth in both treatment groups, along with daily monitoring and recording of mortality.

# LITERATURE CITED

## LITERATURE CITED

**2006.** *Fusarium graminearum* Culture Protocols. University of Kentucky.

**Andreadis, T. G., and R. J. Wolfe. 2010.** Evidence for Reduction of Native Mosquitoes With Increased Expansion of Invasive Ochlerotatus Japonicus Japonicus (Diptera: Culicidae) in the Northeastern United States. Journal of Medical Entomology 47: 43-52.

**Bevins, S. N. 2007.** Establishment and Abundance of a Recently Introduced Mosquito Species Ochlerotatus japonicus (Diptera: Culicidae) in the Southern Appalachians, USA. Journal of Medical Entomology 44: 945-952.

**Brown, D. W., and J. J. Salvo. 1992.** A convenient and safe method to generate a large quantity of Aspergillus parasiticus spores. Fungal Genetics Newsletter 39: 1627-1634.

**Bukhari, T., A. Middelman, C. J. Koenraadt, W. Takken, and B. G. Knols.** Factors affecting fungus-induced larval mortality in Anopheles gambiae and Anopheles stephensi. Malaria journal 9: 22.

**Couch, J. N. 1935.** A New Saprophytic Species of Lagenidium, with Notes on Other Forms. Mycologia 27: 376-387.

**Grooters, A. M., E. C. Hodgin, R. W. Bauer, C. J. Detrisac, N. R. Znajda, and R. C. Thomas. 2003.** Clinicopathologic findings associated with Lagenidium sp. infection in 6 dogs: initial description of an emerging oomycosis. Journal of veterinary internal medicine 17: 637-646.

**Hasan, S., and C. Vago. 1972.** The pathogenicity of Fusarium oxysporum to mosquito larvae. Journal of Invertebrate Pathology 20: 268-271.

**Jeffers, S. N. 2006.** Identifying Species of Phytophthora. Clemson University, Clemson, SC.

**Klich, M. A., and C. voor Schimmelcultures. 2002.** Identification of common Aspergillus species. Centraalbureau voor Schimmelcultures Utrecht.

**Magnoler, A. 1975.** Bioassay of nucleopolyhedrosis virus against larval instars of Malacosoma neustria. Journal of Invertebrate Pathology 25: 343-348.

**Nestrud, L. B., and R. L. Anderson. 1994.** Aquatic safety of Lagenidium giganteum: Effects on freshwater fish and invertebrates. Journal of Invertebrate Pathology 64: 228-233.

**Pettitt, T., A. Wakeham, M. Wainwright, and J. White. 2002.** Comparison of serological, culture, and bait methods for detection of Pythium and Phytophthora zoospores in water. Plant Pathology 51: 720-727.

**Stairs, G. R. 1965.** Quantitative differences in susceptibility to nuclear-polyhedrosis virus among larval Instars of the forest tent caterpillar, Malacosoma disstria (HÃ¼bner). Journal of Invertebrate Pathology 7: 427-429.

**Teetor-Barsch, G. H., and D. W. Roberts. 1983.** Entomogenous Fusarium species. Mycopathologia 84: 3-16.

**Vitoreli, A.** PARP, University of Florida Plant Disease Clinic-GNV mediabook.

# CHAPTER 4: EXAMINATION OF POTENTIAL MECHANISMS OF ACTION OF *ASPERGILLUS NIGER, FUSARIUM OXYSPORUM,* AND *PYTHIUM ULTIMUM* AGAINST LARVAE OF THE MOSQUITO SPECIES *AEDES TRISERIATUS*

## Introduction

It has been shown that many different fungal and oomycete species act as pathogens against mosquitoes. Among those genera that have demonstrated significant pathogenic effects are *Aspergillus* (Moraes et al. 2001, Seye et al. 2009), *Fusarium* (Hasan and Vago 1972), *Coelomomyces* (Couch and Bland 1985), *Lagenidium* (Couch 1935, McCray Jr et al. 1973, Kerwin and Washino 1988), and *Pythium* (Clark et al. 1966, Nnakumusana 1985). These fungi achieve larvicidal or inhibitory effects through a variety of methods, such as mycotoxins production and physical disruption of tissues (Bennett and Klich 2003, Seye et al. 2009).

With any significant effect such as mortality, there is a drive to know not just what the effect is, but how and why it is occurring. With the aforementioned experiments, there was significant mortality observed when *Aedes* larvae were challenged with spores of the fungus *Aspergillus.* There are several different reasons that this could be occurring. First, *Aspergillus* species are known to produce mycotoxins. Having been identified as *Aspergillus niger,* the species of *Aspergillus* used in this study does not produce the potent aflatoxin that some *Aspergillus* species produce, but it does produce ochratoxin A and oxalic acid, both of which can have significant toxic effects in animals (Abarca et al. 1994, Schuster et al. 2002, Bennett and Klich 2003). Alternatively, the *Aspergillus* fungus could be causing mortality in these mosquitoes by sheer physical disruption of host tissue. Fungi in the genus *Aspergillus* have been shown to possess the capability to penetrate the cuticle of larval mosquitoes and thereby cause internal proliferation of hyphae (Seye et al. 2009). The following experiments take a preliminary

approach to determining the mode of action of this fungus against mosquito species. The hypotheses tested were as follows:

**Hypothesis 1:** Mortality and inhibition of development of *Ae. triseriatus* larvae challenged with *Aspergillus* and *Fusarium* isolates is due to a combination of secondary metabolites and direct infection of larvae.

**Hypothesis 2:** *Pythium* isolate effects are due to direct infection of mosquito larvae by motile zoospores, not secondary metabolites.

## Materials and Methods

**Mycotoxin Assay.** Fungal extracellular filtrates were prepared using sterile mung bean broth as a liquid growth medium. Briefly, fungal tissues were added to 250 mL mung bean broth in Erlenmeyer flasks as follows: Untreated controls- no addition, *Aspergillus*- suspended spores from one 50 mL slant of actively growing culture was added, *Fusarium*- one plate of actively growing culture on CYA was cut into approximately 2 cm x 2cm blocks and four blocks were added, *Pythium*- one plate of actively growing culture on 3% cV8A was cut into approximately 2 cm x 2 cm blocks and four blocks were added. Cultures were gently swirled and left to grow on a benchtop at room temperature for two weeks. After two weeks, cultures were filtered through glass fiber filters (Pall Corp, Type A/E, pore size 1.0 µm) to remove hyphal fragments and spores.

Microcosms were set up as follows: 9 mL Milli-Q water, 1 mL fungal extracellular filtrate, and 2 mg Tetramin fish food (Tetra Werke, Melle, Germany) suspended in water were combined in each well of a six-well plate (Costar, Corning Incorporated, Tewksbury, MA). Ten *Ae. triseriatus* larvae were added to each well. There were five wells of ten larvae in each treatment group. Treatment groups were: Untreated control, *Aspergillus*, *Fusarium*, and *Pythium*, with treatment groups differing only in which of the previously described extracellular filtrates was added. The experiment was allowed to run on the benchtop for one week. Larvae were fed 0.2 mg/larva on days 2, 5, and 6. After one week, mortality was evaluated and recorded and larvae were preserved in 3% formalin.

**Microscopic analysis**. Larvae from all previously described experiments were preserved for microscopic analysis. Analysis was conducted using a standard laboratory dissecting microscope. Larvae were examined for obvious injury, external fungal growth, visible internal fungal proliferation, or other unusual features.

## Results

**Mycotoxin Assay.** Survival in the mycotoxin assay was very high overall, at 98%, 88%, 94%, and 94% for *Aspergillus, Fusarium, Pythium*, and untreated controls, respectively (Figure 4.1). There was no significant difference between these levels.

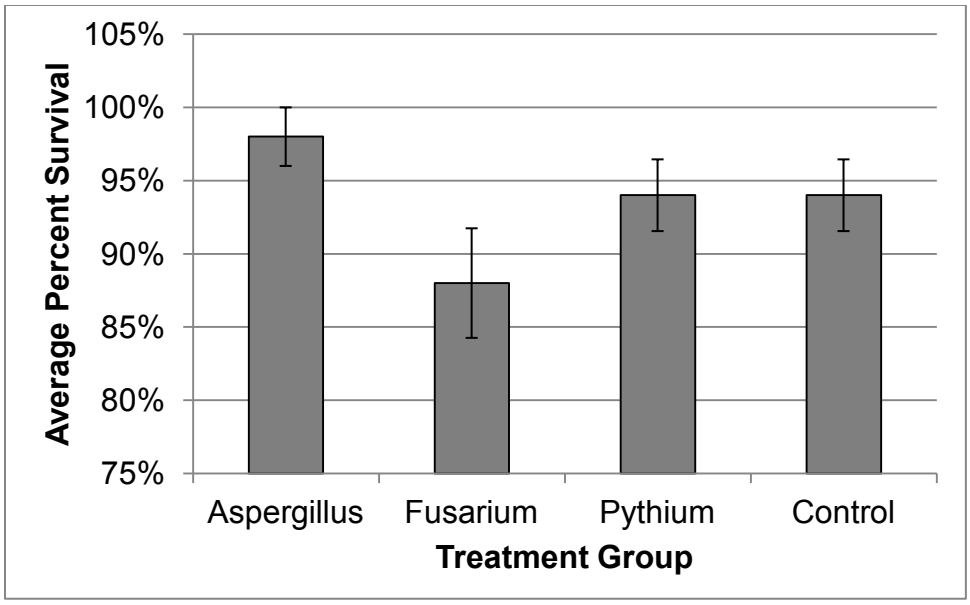


**Figure 4.1.** Survival of *Aedes triseriatus* larvae exposed to fungal extracellular filtrate. No significant differences are present between groups. n=5.

**Microscopic Analysis.** General observations were as follows: in most cases, there was no obvious external fungal growth or sign of infection prior to death. Occasionally, copious filamentous growth could be seen around the anal papillae of moribund larvae (Figure 4.2), although this was not common and tended to occur in those experimental containers where maple leaves were used in place of fungal spore suspensions. Post-mortem analysis revealed very little external growth on cadavers. Growth was observed on a small portion of the sampled larvae, but due to the nature of the experiments it could not be determined based on examination of the intact larvae whether this growth was the cause of death or occurred posthumously. Larvae from an unreported experiment were found to have dark spots on the cuticle, particularly on and around the thorax and between abdominal segments, but these occurred in all treatment groups, and so did not appear to be associated with treatment.

**Figure 4.2.** Photographs taken under a standard dissecting microscope of visible filamentous growth around the anal papillae of moribund *Ae. triseriatus* larvae after growth in maple-based microcosms. Unusual growth marked with arrows.

## Discussion

As a result of the preliminary nature of these studies, a clear conclusion could not be drawn regarding the acceptance or rejection of the hypotheses. It is also not possible to draw any decisive conclusions on whether there are mycotoxins involved in the pathology seen when these *Aedes* mosquitoes are challenged with *Aspergillus,* as there was no significant difference detected between treatment groups. Unfortunately, due to poor larval hatch, this study was restricted to five wells of ten larvae in each treatment group, and treatments at only one dosage per fungal species. In future tests, it would be beneficial to run chemical assays to quantify the amount of known mycotoxins in the media so that dosage can be more accurately judged, and to use a range of dosages.

The microscopic analysis conducted was also of a very preliminary nature. Samples are, at the time of this writing, being processed for histological analysis and analyzed for internal hyphal proliferation and spore germination. These data will hopefully help lend insight into the mode of action of these fungal pathogens. Based on external analysis, it is evident that occasional fungal growth prior to death does occur on the tested mosquito larvae. However, due to the fact that this was generally seen in containers where maple leaves had been used in place of fungal spore solutions, it is possible that the observed growth was due to a fungal species that was not isolated for this work and remains as yet unidentified. Another possibility is that the growth observed on the anal papillae of infected mosquitoes may have been due to the *Pythium* oomycete, which has been shown to infect the anal papillae and wound sites (Clark et al. 1966, Washburn et al. 1988), but the concentrations used in this study were not great enough or the correct infectious stage was not present at the time of

challenge. Also, the species of *Pythium* used in this study was *Pythium ultimum*, which has not previously been shown to have pathogenic effects on invertebrates, but is a well-established plant pathogen which can be isolated from natural samples in Michigan (Martin Chilvers -Personal Communication).

# LITERATURE CITED

## LITERATURE CITED

**Abarca, M., M. Bragulat, G. Castella, and F. Cabanes. 1994.** Ochratoxin A production by strains of Aspergillus niger var. niger. Applied and environmental microbiology 60: 2650-2652.

**Bennett, J. W., and M. A. Klich. 2003.** Mycotoxins. Clinical Microbiology Reviews: 497-516.

**Clark, T. B., W. R. Kellen, J. E. Lindegren, and R. D. Sanders. 1966.** Pythium sp. (Phycomycetes:Pythiales) pathogenic to mosquito larvae. Journal of Invertebrate Pathology 8: 351-354.

**Schuster, E., N. Dunn-Coleman, J. Frisvad, and P. Van Dijck. 2002.** On the safety of Aspergillus niger: a review. Applied microbiology and biotechnology 59: 426-435.

**Seye, F., O. Faye, M. Ndiaye, E. Njie, and J. M. Afoutou. 2009.** Pathogenicity of the fungus, Aspergillus clavatus, isolated from the locust, Oedaleus senegalensis, against larvae of the mosquitoes Aedes aegypti, Anopheles gambiae and Culex quinquefasciatus. Journal of Insect Science 9.

**Washburn, J. A. N. O., D. E. Egerter, J. R. Anderson, and G. A. Saunders. 1988.** Density reduction in larval mosquito (Diptera: Culicidae) populations by interactions between a parasitic ciliate (Ciliophora: Tetrahymenidae) and an opportunistic fungal (Oomycetes: Pythiaceae) parasite. Journal of Medical Entomology 25: 307-314.

# CHAPTER 5: CONCLUSIONS AND DISCUSSION

The overarching goals of this study were to examine an unexpected phenomenon causing increased mortality of larval mosquitoes in the laboratory, to attempt to identify and characterize the etiologic agent or agents for the phenomenon, test the identified and isolated candidate agents against known mosquito species, and to attempt to determine by what method they are achieving the described effect. More specifically, the objectives and hypotheses for these studies were as follows:

**Objective 1:** Assess the presence and magnitude of mosquitocidal or inhibitory effects of isolated fungal/oomycete species against *Ae. triseriatus* larvae.

**Hypothesis 1:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant mosquitocidal effects when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 2:** *Aspergillus, Fusarium,* and *Pythium* isolates will all demonstrate significant inhibition of growth and development when used individually to challenge *Ae. triseriatus* larvae.

**Hypothesis 3:** Mosquitocidal effects will increase with mixed-species inoculum as compared with single-species inoculum.

**Objective 2:** Characterize the mode of action of mosquitocidal fungal/oomycete isolates against *Ae. triseriatus* larvae.

**Hypothesis 1:** Mortality and inhibition of development of *Ae. triseriatus* larvae challenged with *Aspergillus* and *Fusarium* isolates is due to a combination of secondary metabolites and direct infection of larvae.

**Hypothesis 2:** *Pythium* isolate effects are due to direct infection of mosquito larvae by motile zoospores, not secondary metabolites.

Of the isolated fungal species, only *Aspergillus* showed consistent pathogenic effects against either *Aedes triseriatus* or *Aedes japonicus japonicus* larvae. This is contrary to that the hypothesis that all three species would show significant mosquitocidal and developmental effects. Moreover, given its relatively ubiquitous distribution in nature (Schuster et al. 2002), it was expected that mosquitoes of both species would have been exposed to *Aspergillus* more frequently than any of the other species tested and would therefore be less likely to be severely affected by it. It was, in fact, expected that *Pythium* would generate the highest levels of larval mortality, based on the highly pathogenic nature of several species of *Pythium* against both vertebrate and invertebrate animals (Clark et al. 1966, De Cock et al. 1987) and the fact that the closely-related organism *Lagenidium giganteum* has been licensed by the Environmental Protection Agency for use as a larvicidal agent against mosquitoes (USEPA 2001).

It was expected that *Fusarium* would be moderately effective against both species of *Aedes* larvae. This was not the case, as *Fusarium* did not show significant lethal effects in any of the experiments with these larval species. It did, however, produce significant levels of mortality in larvae of the African malaria vector *Anopheles gambiae* (Kisumu strain). This is surprising, as larvae of *Anopheles gambiae* have been shown to be highly competent at mobilizing an immune response called melanization to

protect against these fungal pathogens and thereby surviving infection (Golkar et al. 1993). It is of definite interest that *Fusarium* does show pathogenic/lethal effects against *Anopheles gambiae*, and more studies should be conducted to further explore this relationship.

Unfortunately, very little was able to be concluded regarding the second objective of this study, which was to characterize the mode of action of these fungi against their mosquito hosts based on the mycotoxin assay or external microscopy. Data is currently being collected/analyzed for a more in-depth look at potential physical invasion of the mosquito by the tested fungi, in which samples preserved in 10% neutral buffered formalin were submitted to the Investigative Histopathology Laboratory on the campus of Michigan State University for histological sectioning and staining with hematoxylin and eosin. The slides prepared by the lab are being analyzed for penetration of the cuticle, spore germination within the digestive tract, hyphal penetration and proliferation within the larval body, and fungal growth on the external surface of the cuticle. Larvae from several different treatment groups will be evaluated and compared.

Assays conducted in the field did not show marked mortality of either *Ae. triseriatus* or *Ae. j. japonicus* upon exposure to mixed *Aspergillus* and *Fusarium* spores. This is in direct contrast to the preliminary laboratory bioassays, which were essentially equivalent in their setup, with consistent final concentrations of fungus. The larval density was approximately quadrupled, with 200 larvae per liter in the field microcosm where there had been 10 larvae per 200 mL (50 larvae per liter) in the laboratory microcosms to more closely mimic larval densities observed in the field. It is possible that this contributed to the difference in effect, as the concentration of fungal spores per

larva would then be reduced to one quarter its original amount. Also, there were many environmental factors which could not be controlled in the field, such as temperature, light, rainfall, etc. Any of these could potentially have affected the outcome of these experiments, and it is likely that it was a combination of factors, rather than one in isolation, that ultimately were responsible for the lack of a significant lethal effect in the field that was equivalent to that observed in the laboratory.

Another interesting result of this work is the observation that *Aedes japonicus japonicus* appears to be less susceptible to the tested strain of *Aspergillus* than *Aedes triseriatus*, which could contribute to a possible competitive advantage in the wild. To date, little has been published regarding competition between these two species, but it has been suggested that *Ae. j. japonicus* may be able to outcompete *Ae. triseriatus* in the wild, leading to decreased occurrence of the latter species in their former habitats (Bevins 2007, Andreadis and Wolfe 2010). In addition to the intrinsic ecological importance of invasive species biology, this relationship may be of additional public health importance, as larval competition has been shown to affect the competence of adult mosquitoes as vectors of disease and longevity of the adult mosquito (Alto et al. 2005, Alto 2011).

There are many directions that can be taken as future explorations of these complex relationships are planned. For example, it would be of value to more specifically determine the dosages at which these fungi have demonstrable action against the development and survival of *Aedes triseriatus* larvae. Perhaps more interesting would be to look at the potential competitive advantage of *Aedes japonicus japonicus* when compared with *Aedes triseriatus* after exposure to these pathogens.

There is little known about the competitive interactions of these two species. The potential for *Ae. j. japonicus* to spread as an invasive species and develop as a disease vector makes this an intriguing area of research for future studies. Finally, it would be of great value to further examine the mode of action of these fungal species, particularly *Aspergillus niger*, which was the most effective at killing *Aedes* mosquitoes.

# LITERATURE CITED

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**2006.** *Fusarium graminearum* Culture Protocols. University of Kentucky.

**Abarca, M., M. Bragulat, G. Castella, and F. Cabanes. 1994.** Ochratoxin A production by strains of Aspergillus niger var. niger. Applied and environmental microbiology 60: 2650-2652.

**Alpert, M. E., M. S. R. Hutt, G. N. Wogan, and C. S. Davidson. 1971.** Association between aflatoxin content of food and hepatoma frequency in Uganda. Cancer 28: 253-260.

**Alto, B. W. 2011.** Interspecific Larval Competition between Invasive Aedes japonicus and Native Aedes triseriatus (Diptera: Culicidae) and Adult Longevity. Journal of Medical Entomology 48: 232-242.

**Alto, B. W., L. P. Lounibos, S. Higgs, and S. A. Juliano. 2005.** Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology 86: 3279-3288.

**Andrade, C. F. S. 1993.** *Metarhizium anisopliae* (Metsch.) Sorok.: Potential to recycle in mosquito larvae.

**Andreadis, T. G., and R. J. Wolfe. 2010.** Evidence for Reduction of Native Mosquitoes With Increased Expansion of Invasive Ochlerotatus Japonicus Japonicus (Diptera: Culicidae) in the Northeastern United States. Journal of Medical Entomology 47: 43-52.

**Anthony, D. W., H. C. Chapman, and E. I. Hazard. 1971.** Scanning electron microscopy of the sporangia of species of Coelomomyces (Blastocladiales: Coelomomycetaceae). Journal of Invertebrate Pathology 17: 395-403.

**Arrivillaga, J., and R. Barrera. 2004.** Food as a limiting factor for Aedes aegypti in water-storage containers. Journal of Vector Ecology 29: 11-20.

**Avellis, F. O., A. Bartolino, P. Benedetti, L. H. Kramer, P. Mora, and F. Rivasi. 2011.** A case of human conjunctival dirofilariosis by Dirofilaria immitis in Italy. Vector-Borne and Zoonotic Diseases 11: 451+.

**Ballard, E., and F. Knapp. 1984.** Occurrence of the fungus Verticillium lecanii on a new host species: Aedes triseriatus (Diptera: Culicidae). Journal of Medical Entomology 21: 751-751.

**Bennett, J. W., and M. A. Klich. 2003.** Mycotoxins. Clinical Microbiology Reviews: 497-516.

**Bevins, S. N. 2007.** Establishment and Abundance of a Recently Introduced Mosquito Species Ochlerotatus japonicus (Diptera: Culicidae) in the Southern Appalachians, USA. Journal of Medical Entomology 44: 945-952.

**Borneman, J., and R. J. Hartin. 2000.** PCR primers that amplify fungal rRNA genes from environmental samples. Applied and environmental microbiology 66: 4356-4360.

**Borucki, M. K., B. J. Kempf, B. J. Blitvich, C. D. Blair, and B. J. Beaty. 2002.** La Crosse virus: replication in vertebrate and invertebrate hosts. Microbes and Infection 4: 341-350.

**Briegel, H. 1990.** Metabolic relationship between female body size, reserves, and fecundity of Aedes aegypti. Journal of Insect Physiology 36: 165-172.

**Brown, D. W., and J. J. Salvo. 1992.** A convenient and safe method to generate a large quantity of Aspergillus parasiticus spores. Fungal Genetics Newsletter 39: 1627-1634.

**Bukhari, T., A. Middelman, C. J. Koenraadt, W. Takken, and B. G. Knols.** Factors affecting fungus-induced larval mortality in Anopheles gambiae and Anopheles stephensi. Malaria journal 9: 22.

**CDC. 2009.** La Crosse Encephalitis. *In* C. f. D. C. a. Prevention [ed.], Atlanta, GA.

**CDC. 2012.** Mosquito species in which West Nile virus has been detected, United States, 1999-2012.

**Chamberlain, R. W., R. K. Bikes, D. B. Nelson, and W. D. Sudia. 1954.** Studies on the North American Arthropod-borne Encephalitides. American Journal of Epidemiology 60: 278-285.

**Chapman, H. C. 1974.** Biological control of mosquito larvae. Annual Review of Entomology 19: 33-59.

**Clark, T. B., W. R. Kellen, J. E. Lindegren, and R. D. Sanders. 1966.** Pythium sp. (Phycomycetes:Pythiales) pathogenic to mosquito larvae. Journal of Invertebrate Pathology 8: 351-354.

**Couch, J. N. 1935.** A New Saprophytic Species of Lagenidium, with Notes on Other Forms. Mycologia 27: 376-387.

**Couch, J. N., and C. E. Bland. 1985.** The genus Coelomomyces. Academic Press.

**Dâ€™Mello, J. P. F., C. M. Placinta, and A. M. C. Macdonald. 1999.** Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology 80: 183-205.

**Dashiell, G. F. 1961.** A Case of Dirofilariasis Involving the Lung. The American journal of tropical medicine and hygiene 10: 37-38.

**De Cock, A., L. Mendoza, A. Padhye, L. Ajello, and L. Kaufman. 1987.** Pythium insidiosum sp. nov., the etiologic agent of pythiosis. Journal of clinical microbiology 25: 344.

**Debboun, M., T. J. Green, L. M. Rueda, and R. D. Hall. 2005.** Relative abundance of tree hole-breeding mosquitoes in Boone County, Missouri, USA, with emphasis on the vector potential of aedes triseriatus for canine heartworm, Dirofilaria immitis (Spirurida: Filariidae). Journal of the American Mosquito Control Association 21: 274-278.

**Eaton, D. L., and E. P. Gallagher. 1994.** Mechanisms of Aflatoxin Carcinogenesis. Annual Review of Pharmacology and Toxicology 34: 135-172.

**Eriksen, G. S. I., and H. Pettersson. 2004.** Toxicological evaluation of trichothecenes in animal feed. Animal Feed Science and Technology 114: 205-239.

**Fish, D., and S. R. Carpenter. 1982.** Leaf Litter and Larval Mosquito Dynamics in Tree-Hole Ecosystems. Ecology 63: 283-288.

**Gaastra, W., L. J. A. Lipman, A. W. A. M. De Cock, T. K. Exel, R. B. G. Pegge, J. Scheurwater, R. Vilela, and L. Mendoza.** Pythium insidiosum: An overview. Veterinary microbiology 146: 1-16.

**Gauld, L., R. Hanson, W. Thompson, and S. Sinha. 1974.** Observations on a natural cycle of La Crosse virus (California group) in Southwestern Wisconsin. The American journal of tropical medicine and hygiene 23: 983-992.

**Gaulin, E., A. Bottin, and B. Dumas. 2010.** Sterol biosynthesis in oomycete pathogens. Plant signaling & behavior 5: 258-260.

**Geraats, B. P. J., P. A. H. M. Bakker, and L. Van Loon. 2002.** Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and Arabidopsis thaliana. Molecular plant-microbe interactions 15: 1078-1085.

**Gerhardt, R. R., K. L. Gottfried, C. S. Apperson, B. S. Davis, P. C. Erwin, A. B. Smith, N. A. Panella, E. E. Powell, and R. S. Nasci. 2001.** First isolation of La Crosse virus from naturally infected Aedes albopictus. Emerging infectious diseases 7: 807.

**Golkar, L., R. A. Lebrun, H. Ohayon, P. Gounon, B. Papierok, and P. T. Brey. 1993.** Variation of Larval Susceptibility to Lagenidium giganteum in Three Mosquito Species. Journal of Invertebrate Pathology 62: 1-8.

**Gonczol, J., and A. Revay. 2003.** Treehole fungal communities: aquatic, aero-aquatic and dematiaceous hyphomycetes. Fungal diversity 12: 19-34.

**Govindarajan, M., A. Jebanesan, and D. Reetha. 2005.** Larvicidal effect of extracellular secondary metabolites of different fungi against the mosquito, Culex quinquefasciatus Say. Tropical biomedicine 22: 1-3.

**Grimstad, P. R., and L. D. Haramis. 1984.** Aedes triseriatus (Diptera: Culicidae) and La Crosse virus III. Enhanced oral transmission by nutrition-deprived mosquitoes. Journal of Medical Entomology 21: 249-256.

**Grimstad, P. R., and E. D. Walker. 1991.** Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and transmission. Journal of Medical Entomology 28: 378-386.

**Grooters, A. M., E. C. Hodgin, R. W. Bauer, C. J. Detrisac, N. R. Znajda, and R. C. Thomas. 2003.** Clinicopathologic findings associated with Lagenidium sp. infection in 6 dogs: initial description of an emerging oomycosis. Journal of veterinary internal medicine 17: 637-646.

**Hasan, S., and C. Vago. 1972.** The pathogenicity of Fusarium oxysporum to mosquito larvae. Journal of Invertebrate Pathology 20: 268-271.

**Ho, W. C., and W. H. Ko. 1997.** A simple method for obtaining single-spore isolates of fungi. Botanical Bulletin of Academia Sinica 38.

**Hollidge, B., F. Gonzalez-Scarano, and S. Soldan. 2010.** Arboviral Encephalitides: Transmission, Emergence, and Pathogenesis. Journal of Neuroimmune Pharmacology 5: 428-442.

**Intermill, R. 1973.** Development of Dirofilaria immitis in Aedes triseriatus Say. Mosquito News 33: 176-181.

**Jeffers, S. N. 2006.** Identifying Species of Phytophthora. Clemson University, Clemson, SC.

**Jones, T. F., A. S. Craig, R. S. Nasci, L. E. Patterson, P. C. Erwin, R. R. Gerhardt, X. T. Ussery, and W. Schaffner. 1999.** Newly recognized focus of La Crosse encephalitis in Tennessee. Clinical infectious diseases 28: 93-97.

**Kaufman, M. G., S. Chen, and E. D. Walker. 2008.** Leaf-associated bacterial and fungal taxa shifts in response to larvae of the tree hole mosquito, Ochlerotatus triseriatus. Microbial Ecology 55: 673-684.

**Kaufman, M. G., S. N. Bland, M. E. Worthen, E. D. Walker, and M. J. Klug. 2001.** Bacterial and fungal biomass responses to feeding by larval Aedes triseriatus (Diptera: Culicidae). Journal of Medical Entomology 38: 711-719.

**Kaufman, M. G., W. Goodfriend, A. Kohler-Garrigan, E. D. Walker, and M. J. Klug. 2002.** Soluble nutrient effects on microbial communities and mosquito production in Ochlerotatus triseriatus habitats. Aquatic Microbial Ecology 29: 73-88.

**Kaufman, M. G., K. S. Pelz-Stelinski, D. A. Yee, S. A. Juliano, P. H. Ostrom, and E. D. Walker. 2010.** Stable isotope analysis reveals detrital resource base sources of the tree hole mosquito, Aedes triseriatus. Ecological entomology 35: 586-593.

**Kerwin, J. L., and R. K. Washino. 1988.** Field Evaluation of Lagenidium giganteum (Oomycetes: Lagenidiales) and Description of a Natural Epizootic Involving a New Isolate of the Fungus. Journal of Medical Entomology 25: 452-460.

**Kerwin, J. L., D. A. Dritz, and R. K. Washino. 1990.** Confirmation of the safety of Lagenidium giganteum (Oomycetes: Lagenidiales) to mammals. Journal of economic entomology 83: 374-376.

**Kitching, R. L. 1971.** An Ecological Study of Water-Filled Tree-Holes and their Position in the Woodland Ecosystem. Journal of Animal Ecology 40: 281-302.

**Klich, M. A., and C. voor Schimmelcultures. 2002.** Identification of common Aspergillus species. Centraalbureau voor Schimmelcultures Utrecht.

**Lodge, D. J., P. J. Fisher, and B. C. Sutton. 1996.** Endophytic Fungi of Manilkara bidentata Leaves in Puerto Rico. Mycologia 88: 733-738.

**Luz, C., M. Netto, and L. Rocha. 2007.** In vitro susceptibility to fungicides by invertebrate-pathogenic and saprobic fungi. Mycopathologia 164: 39-47.

**Magnoler, A. 1975.** Bioassay of nucleopolyhedrosis virus against larval instars of Malacosoma neustria. Journal of Invertebrate Pathology 25: 343-348.

**Martin, F. N., and J. E. Loper. 1999.** Soilborne plant diseases caused by Pythium spp.: ecology, epidemiology, and prospects for biological control. Critical Reviews in Plant Sciences 18: 111-181.

**McCorkindale, N. J., S. A. Hutchinson, B. A. Pursey, W. T. Scott, and R. Wheeler. 1969.** A comparison of the types of sterol found in species of the saprolegniales and leptomitales with those found in some other phycomycetes. Phytochemistry 8: 861-867.

**McCray Jr, E., C. Umphlett, and R. Fay. 1973.** Laboratory studies on a new fungal pathogen of mosquitoes. Mosquito News 33: 54-60.

**McJunkin, J. E., R. Khan, C. Emily, D. L. Parsons, L. L. Minnich, R. G. Ashley, and T. F. Tsai. 1997.** Treatment of severe La Crosse encephalitis with intravenous ribavirin following diagnosis by brain biopsy. Pediatrics 99: 261-267.

**McJunkin, J. E., E. C. Reyes, J. E. Irazuzta, M. J. Caceres, R. R. Khan, L. L. Minnich, K. D. Fu, G. D. Lovett, T. Tsai, and A. Thompson. 2001.** La Crosse Encephalitis in Children. New England Journal of Medicine 344: 801-807.

**McLintock, J. 1978.** Mosquito-Virus Relationships of American Encephalitides. Annual Review of Entomology 23: 17-37.

**Merrill, J. R., J. Otis, W. D. Logan Jr, and M. B. Davis. 1980.** The Dog Heartworm (*Dirofilaria immitis*) in Man. Journal of the American Medical Association 243: 1066-1068.

**Molaei, G., A. Farajollahi, J. J. Scott, R. Gaugler, and T. G. Andreadis. 2009.** Human Bloodfeeding by the Recently Introduced Mosquito, Aedes japonicus japonicus, and Public Health Implications. Journal of the American Mosquito Control Association 25: 210-214.

**Moorhouse, D. 1978.** Dirofilaria immitis: A cause of human intra-ocular infection. Infection 6: 192-193.

**Moraes, A., G. Costa, M. Barcellos, R. Oliveira, and P. Oliveira. 2001.** The entomopathogenic potential of Aspergillus spp. in mosquitoes vectors of tropical diseases. J. Basic Microbiol 41: 45-49.

**Nadeau, M., and J. Boisvert. 1994.** Larvicidal activity of the entomopathogenic fungus Tolypocladium cylindrosporum (Deuteromycotina: Hyphomycetes) on the mosquito Aedes triseriatus and the black fly Simulium vittatum (Diptera: Simuliidae). Journal of the American Mosquito Control Association 10: 487-491.

**Naksathit, A. T., J. D. Edman, and T. W. Scott. 1999.** Amounts of Glycogen, Lipid, and Sugar in Adult Female Aedes aegypti (Diptera: Culicidae) Fed Sucrose. Journal of Medical Entomology 36: 8-12.

**Nasci, R., C. Moore, B. Biggerstaff, N. Panella, H. Liu, N. Karabatsos, B. Davis, and E. Brannon. 2000.** La Crosse encephalitis virus habitat associations in Nicholas County, West Virginia. Journal of Medical Entomology 37: 559-570.

**Nestrud, L. B., and R. L. Anderson. 1994.** Aquatic safety of Lagenidium giganteum: Effects on freshwater fish and invertebrates. Journal of Invertebrate Pathology 64: 228-233.

**Nnakumusana, E. 1985.** Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to Aspergillus parasiticus and its effects on fecundity and longevity of mosquitoes exposed to sporal infections in larval stages. Current Science 54: 1221.

**Onken, H., S. B. Moffett, and D. F. Moffett. 2004.** The anterior stomach of larval mosquitoes (Aedes aegypti): effects of neuropeptides on transepithelial ion transport and muscular motility. Journal of Experimental Biology 207: 3731-3739.

**Paulson, S. L., and W. A. Hawley. 1991.** Effect of body size on the vector competence of field and laboratory populations of *Aedes triseriatus* for La Crosse Virus. Journal of the American Mosquito Control Association 7: 170-175.

**Pettitt, T., A. Wakeham, M. Wainwright, and J. White. 2002.** Comparison of serological, culture, and bait methods for detection of Pythium and Phytophthora zoospores in water. Plant Pathology 51: 720-727.

**Peyton, E., S. R. Campbell, T. M. Candeletti, M. Romanowski, and W. J. Crans. 1999.** Aedes (Finlaya) japonicus japonicus (Theobald), a new introduction into the United States. DTIC Document.

**Placinta, C. M., J. P. F. D'Mello, and A. M. C. Macdonald. 1999.** A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. Animal Feed Science and Technology 78: 21-37.

**Prakash, S., G. Singh, N. Soni, and S. Sharma. 2010.** Pathogenicity of Fusarium oxysporum against the larvae of Culex quinquefasciatus (Say) and Anopheles stephensi (Liston) in laboratory. Parasitology research 107: 651-655.

**Rahuman, A. A., G. Gopalakrishnan, P. Venkatesan, and K. Geetha. 2008.** Larvicidal activity of some Euphorbiaceae plant extracts against Aedes aegypti and Culex quinquefasciatus (Diptera: Culicidae). Parasitology research 102: 867-873.

**Rust, R. S., W. H. Thompson, C. G. Matthews, B. J. Beaty, and R. W. Chun. 1999.** Topical Review: La Crosse and Other Forms of California Encephalitis. Journal of child neurology 14: 1-14.

**Sardelis, M. R., M. J. Turell, and R. G. Andre. 2002a.** Laboratory Transmission of La Crosse Virus by Ochlerotatus j. japonicus (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

**Sardelis, M. R., D. J. Dohm, B. Pagac, R. G. Andre, and M. J. Turell. 2002b.** Experimental Transmission of Eastern Equine Encephalitis Virus by Ochlerotatus j. japonicus (Diptera: Culicidae). Journal of Medical Entomology 39: 480-484.

**Schaffner, F., C. Kaufmann, D. Hegglin, and A. Mathis. 2009.** The invasive mosquito Aedes japonicus in Central Europe. Medical and Veterinary Entomology 23: 448-451.

**Schurko, A., L. Mendoza, A. W. A. M. de Cock, and G. R. Klassen. 2003.** Evidence for geographic clusters: Molecular genetic differences among strains of Pythium insidiosum from Asia, Australia and the Americas are explored. Mycologia 95: 200-208.

**Schuster, E., N. Dunn-Coleman, J. Frisvad, and P. Van Dijck. 2002.** On the safety of Aspergillus niger: a review. Applied microbiology and biotechnology 59: 426-435.

**Seitz, L. M., D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard. 1979.** Ergosterol as a measure of fungal growth. *Phytopathology* 69: 1202-1203.

**Seye, F., O. Faye, M. Ndiaye, E. Njie, and J. M. Afoutou. 2009.** Pathogenicity of the fungus, Aspergillus clavatus, isolated from the locust, Oedaleus senegalensis, against larvae of the mosquitoes Aedes aegypti, Anopheles gambiae and Culex quinquefasciatus. Journal of Insect Science 9.

**Seymour, R. L. 1984.** Leptolegnia chapmanii, an oomycete pathogen of mosquito larvae. Mycologia: 670-674.

**Sokol, D. K., M. B. Kleiman, and B. P. Garg. 2001.** LaCrosse viral encephalitis mimics herpes simplex viral encephalitis. Pediatric neurology 25: 413-415.

**Soldan, S. S., and F. Gonzalez-Scarano. 2005.** Emerging infectious diseases: the Bunyaviridae. Journal of neurovirology 11: 412-423.

**Stairs, G. R. 1965.** Quantitative differences in susceptibility to nuclear-polyhedrosis virus among larval Instars of the forest tent caterpillar, Malacosoma disstria (HÃ¼bner). Journal of Invertebrate Pathology 7: 427-429.

**Su, X. 2008.** A Report of the Mosquito Host Range of Pythium guiyangense Su.

**Sudia, W., V. Newhouse, C. Calisher, and R. Chamberlain. 1971.** California group arboviruses: Isolations from mosquitoes in North America. Mosq. News 31: 576-600.

**Tatum, L. M., J. M. Pacy, K. S. Frazier, J. F. Weege, C. A. Baldwin, G. A. Hullinger, G. D. Bossart, and N. H. Altman. 1999.** Canine LaCrosse viral meningoencephalomyelitis with possible public health implications. Journal of Veterinary Diagnostic Investigation 11: 184-188.

**Teetor-Barsch, G. H., and D. W. Roberts. 1983.** Entomogenous Fusarium species. Mycopathologia 84: 3-16.

**Thompson, W., and B. Beaty. 1977.** Venereal transmission of La Crosse (California encephalitis) arbovirus in Aedes triseriatus mosquitoes. Science 196: 530-531.

**Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001.** Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. Journal of Medical Entomology 38: 130-134.

**Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. O'guinn, T. G. Andreadis, and J. A. Blow. 2005.** An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 42: 57-62.

**USEPA. 2001.** *Lagenidium giganteum* (129084) Fact Sheet. *In* U. S. E. P. Agency [ed.].

**USEPA. 2011.** Notice of Receipt of Requests To Voluntarily Cancel Certain Pesticide Registrations. Federal Register 76: 47579-47582.

**Utz, J. T., C. S. Apperson, and E. J. Dietz. 2005.** Social impacts of la crosse encephalitis in North Carolina. Human organization 64: 135-146.

**Vitoreli, A.** PARP, University of Florida Plant Disease Clinic-GNV mediabook.

**Walker, E. D., D. L. Lawson, R. W. Merritt, W. T. Morgan, and M. J. Klug. 1991.** Nutrient Dynamics, Bacterial Populations, and Mosquito Productivity in Tree Hole Ecosystems and Microcosms. Ecology 72: 1529-1546.

**Walker, N. 1992.** The Eastern Treehole Mosquito: *Aedes triseriatus*. Wing Beats 3: 1.

**Wang, L.-Y., M. Hatch, C.-J. Chen, B. Levin, S.-L. You, S.-N. Lu, M.-H. Wu, W.-P. Wu, L.-W. Wang, Q. Wang, G.-T. Huang, P.-M. Yang, H.-S. Lee, and R. M. Santella. 1996.** Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. International Journal of Cancer 67: 620-625.

**Washburn, J. A. N. O., D. E. Egerter, J. R. Anderson, and G. A. Saunders. 1988.** Density reduction in larval mosquito (Diptera: Culicidae) populations by interactions between a parasitic ciliate (Ciliophora: Tetrahymenidae) and an opportunistic fungal (Oomycetes: Pythiaceae) parasite. Journal of Medical Entomology 25: 307-314.

**Watts, D. M., C. D. Morris, R. E. Wright, G. R. DeFoliart, and R. P. Hanson. 1972.** Transmission of LaCrosse Virus (California Encephalitis Group) by the mosquito *Aedes triseriatus*. Journal of Medical Entomology 9: 125-127.

**Watts, D. M., W. H. Thompson, T. M. Yuill, G. R. DeFoliart, and R. P. Hanson. 1974.** Overwintering of La Crosse virus in Aedes triseriatus. The American journal of tropical medicine and hygiene 23: 694.

**Werner, D., and H. Kampen. 2013.** The further spread of Aedes japonicus japonicus (Diptera, Culicidae) towards northern Germany. Parasitology research 112: 3665-3668.

**Whisler, H. C., S. L. Zebold, and J. A. Shemanchuk. 1974.** Alternate host for mosquito parasite Coelomomyces. Nature 251: 715-716.

**Whitley, R. J., and J. W. Gnann. 2002.** Viral encephalitis: familiar infections and emerging pathogens. The Lancet 359: 507-513.

**Williams, M. C., and R. W. Lichtwardt. 1972.** Infection of Aedes aegypti larvae by axenic cultures of the fungal genus Smittium (Trichomycetes). American Journal of Botany: 189-193.

**Yee, D. A., M. G. Kaufman, and S. A. Juliano. 2007.** The significance of ratios of detritus types and micro-organism productivity to competitive interactions between aquatic insect detritivores. Journal of Animal Ecology 76: 1105-1115.

**Yuill, T. M. 1984.** The Role of Forest Mammals in La Crosse Virus Epidemiology. Proceedings of the International Conference of Wildlife Disease Association: 23-24.

**Zaim, M., K. Baker, and H. Newson. 1979.** Natural infection of the tree hole breeding mosquito Aedes triseriatus (Diptera: Culicidae) with the fungus Funicularius triseriatus. Journal of Invertebrate Pathology 34: 199-202.