# I. MICROBIAL/GENETIC STRATEGIES FOR MOSQUITO CONTROL

**Wildlife # 4**

Program Managers: A. M. Fallon and T. J. Kurtti
Department of Entomology
University of Minnesota
625-3728; 624-4740

A. ML 1991, Ch. 254, Art 1, Sec. 14, Subd. 9 (c) Appropriation: $150,000
Balance: $ 0

This appropriation is to the University of Minnesota, Department of Entomology, to enhance mosquito control by development of microbial agents that are environmentally safe and specific for mosquitoes.

B. Compatible data: Not applicable

C. Match Requirement: Not applicable

## II. NARRATIVE:

### A. STATEMENT OF THE PROBLEM

Mosquitoes seriously impact the economy and resources of Minnesota. Mosquito biting behavior restricts recreational activities and in some instances results in transmission of LaCrosse and Western Encephalitis viruses. Although effective reduction of mosquito populations using relatively non-toxic chemicals such as the insect growth regulator methoprene (Altosid) can be accomplished in the Metro area, equivalent control in outlying areas is not economically feasible.

### B. IMPORTANCE:

Mosquitoes have an excellent track record for evolving resistance to chemical control agents. For example, only a generation ago, scientists were optimistic that malaria could be eliminated through extensive use of DDT and other chemical pesticides. In spite of initial successes with this approach, malaria has regained its status among the most prevalent diseases on earth (Klayman, 1989). Current strategies for mosquito control, based on insect growth regulators such as Altosid, are a practical outgrowth of more than two decades of basic research on the insect hormones and their effects on growth and reproduction. Although demonstrated resistance to the insect growth regulators is at present limited to only a few species (Shemshedini and Wilson, 1990), it remains prudent to anticipate increased instances of resistance, and therefore to begin to assess alternative control strategies. Environmental concerns focus attention on the naturally-occurring microorganisms which are pathogenic for mosquitoes and which might be developed into effective control agents by judicious selection for desirable properties. Within this context, the goal of the present proposal is to develop microbial control agents whose use may enhance ongoing control efforts and contribute to the underlying basic



knowledge that will be important for development of the next generation of mosquito control practices. An important component of our research approach will be to develop strains that incorporate desirable microbial properties (virulence, specificity) with desirable physiological effects (mortality, decreased feeding or fecundity) on the target mosquitoes.

### C. EXTENT OF THE PROBLEM:

Mosquito control is a worldwide problem. Although for most Minnesotans, mosquitoes are primarily of nuisance potential, for the few individuals who contract mosquito-borne encephalitis, mosquitoes may have devastating impact. Mosquito-transmitted disease is increasing worldwide, due to the resistance of mosquitoes to conventional control agents, and to the increasing resistance of the pathogens vectored by mosquitoes to therapeutic drugs. The development of effective vaccines for mosquito-transmitted diseases has been disappointingly slow. In consequence, the World Health Organization is putting increased emphasis on development of novel control strategies based on mosquito genetics and physiology. Our laboratories are uniquely equipped to explore new strategies for mosquito control, and to train today's students in the relevant technologies. In these efforts, we will draw upon advances made in other Federal and State-sponsored projects ongoing in our laboratories, in the anticipation of developing approaches that will be particularly useful in Minnesota.

## III. OBJECTIVE: TO SELECT AND IMPROVE MICROBIAL PATHOGENS OF MOSQUITOES. This is an integrated study having a single objective with two supporting objectives:

### A. Develop new and improved strains of pathogenic microsporidia for use in microbial control of mosquitoes.

A. I. Narrative: The focus of this supporting objective is to isolate strains of microsporidia having features useful to the control of mosquitoes in Minnesota, especially the most prevalent mosquito, *Aedes vexans*. In spite of successes with agricultural pests, few microbial control agents specific for mosquitoes are commercially available. Insect pathologists have identified a number of microorganisms that specifically infect and kill mosquitoes. Among those with particular potential are the microsporidia, a group of spore-forming protozoa (Brooks, 1988). New and more effective strains are needed. As has been done, for example, in the development of vaccines, strains with useful biological properties can be obtained by selection in the laboratory.

A. 2. Procedures: We will develop technologies to isolate and manipulate strains of *Nosema algerae* as our choice of a candidate microsporidian with potential for mosquito control. Two biological mechanisms that may contribute to improved strains of *N. algerae* include virulence and differential expression of essential genes, whose activities may provide approaches for phenotypic selection. A major thrust of the research will be to develop methods to isolate, from mixed populations, natural variants with properties such as exceptional ability to infect the mosquito, to disseminate in nature or to survive over the winter.

Microsporidia are obligate intracellular microorganisms, which need to grow in live cells (or organisms). The initial approach to isolation of strains will be to establish conditions for maintenance of the pathogen in mosquito cell culture, using techniques that have already been developed for producing microsporidia in cells from lepidopteran pests (Kurtti et. al., 1990). Briefly, spores will be mass-produced in mosquitoes, purified, activated, and used to infect cultured mosquito cells (Kurtti et al., 1990). Microsporidia that will be recovered from the infected cell culture will represent a **genetically diverse population**, which will express various selectable traits. Our goal will be to isolate from this mixed population, **individuals** that express desired traits, using standard microbiological approaches. Individuals with desirable traits can then be used to **establish genetically uniform populations**, or clones, by culturing the individual spores in isolation. This can be achieved by diluting the stock population of microsporidia to the extent that there would be fewer than one individual per ml of culture fluid. Mosquito cells in petri plates would be inoculated with 1.0 ml of diluted microsporidia, and observed for signs of infection. Microsporidia recovered from infected cultures would represent clonal populations, each of which originated from a single individual. In addition to the limiting dilution technique, we will also use a newly-developed plaque assay procedure (Kurtti, Ross, and Munderloh, unpublished). Mosquito cells infected using low spore to cell ratios (less than 1) will be seeded at low dilutions onto uninfected cell layers and overlaid with medium solidified with agarose. Colonies (plaques) of infected cells differing (as a result of infection) in size and/or morphology from one another will be recovered and tested for mosquito pathogenicity. In initial studies, we will compare 10 - 20 clones of *N. algerae* biologically, using mosquitoes (see objective B, below) and mosquito cell culture bioassays. Protein and DNA characterization will be done to distinguish variant strains from one another and to aid their detection and monitoring in future field trials.

Mosquitoes (*Aedes vexans* and *Aedes aegypti*) will be reared in the laboratory and used to evaluate the effects of individual *N. algerae* clones on larval survival and on adult emergence. First, dosage experiments will be carried out to evaluate the infectivity of the various *Nosema* clones recovered as described above. Mosquito larvae will be treated with various numbers of *Nosema* spores, and survival to the pupal and adult stages will be measured. Delays in developmental rates will be noted and correlated with their effects on the surviving adult mosquitoes. For example, we plan to determine whether adults that develop from infected larvae have normal longevity, feeding behavior, and reproductive success. These data are technically easy to obtain, and will provide important baseline information. Probit analysis and analysis of variance will be used to evaluate differences in the effects of various *Nosema* clones on the mosquito. This statistical evaluation will enable us **to rank our genetically uniform clones in terms of an important property: virulence to the intended target organism.**

Candidate strains will further need to be evaluated for their potential ability to survive in the wild, although actual release of organisms resulting from our studies will not be pursued until substantial testing has been done, and Federal and State guidelines met. Duplication of many environmental conditions can be accomplished in the lab using temperature and humidity-controlled growth chambers and freezers. Tolerance to freezing and dehydration will be evaluated as a function of time of storage, over a period encompassing the duration of this project



and any extensions thereof. Successful storage and tolerance to potential environmental factors will be correlated with virulence. Clearly, candidates that are unable to survive prolonged freezing, or a number of freeze-thaw cycles, will not be useful for permanent control of mosquitoes in Minnesota, but they may be useful in field tests, where "containment" might be desirable, or for eventual field application in milder climates. If necessary, microsporidia clones that are selected for virulence will be subjected to a second cycle of clonal isolation and selection for ability to tolerate appropriate conditions of temperature and humidity.

Environmental survival will also be evaluated by mimicking aquatic habitats in which microsporidia will eventually be used. Water and associated material from typical mosquito habitats will be used for rearing laboratory mosquitoes, and survival of microsporidia, and virulence to the mosquito, will be tested. Using such a system, virulence to other aquatic insects and arthropods can also be assessed. To ensure accuracy of results, on the order of 500 mosquito larvae will be used in such tests, and results will be replicated under a range of temperature conditions based on water temperatures in mosquito habitats at different time of the breeding season.

Strains of *Nosema* representing low, moderate, and high levels of virulence will be further characterized to identify biochemical properties that will enable us to measure long-term stability of these strains. Proteins will be characterized using native and denaturing polyacrylamide gel electrophoresis. For this procedure, microsporidia will be lysed in a solution containing buffer and detergent (denaturing gels only), and applied to a gel matrix. Under the influence of an electric current, proteins will migrate through the gel matrix according to size or charge, producing a pattern that has diagnostic features. Usually, the size of a protein (molecular weight) or abundance of unique proteins (that may be correlated with a desired property) provide useful biochemical markers. Loss of such a marker during maintenance of a strain would suggest loss of the desired property.

More sophisticated methods for tracking these strains will include analysis of chromosomal DNA. Chromosomes from microsporidia will be analyzed by pulsed field and horizontal agarose gel electrophoresis (Munderloh et al., 1990). Such analyses are now routine, and will be essential for publication of our results in high-visibility, peer-reviewed journals. The biochemical properties that we establish in this aspect of the work will enable us to monitior the stability of strains over time, and to evaluate any genetic changes that occur during cyclical propagation of the microsporidia in cell culture, in the mosquito, and eventually in the environment.

A. 3 Budget:

a. Amount budgeted: $75,000
b. Balance: $ 0

A. 4. Timeline for Products/Tasks

| | 7/91 | 1/92 | 6/92 | 1/93 | 6/93 |
|---|---|---|---|---|---|
| > Isolate strains | ********** | | | | |
| >Characterize strains | | ********** | | | |
| >Identify strains useful for mosquito control | | | ********** | | |
| >Final report and manuscript publication | | | | | ********** |

A. 5. Status: Period 1 (7/91-12/91). We worked on improving the procedures for rearing A. vexans in the laboratory. Methods to improve feeding and egg laying by adult females of our established *A. vexans* colony were developed. These improvements will be important for the year round rearing of A. vexans and when we need to grow large numbers of larvae for our bioassays. In collaboration with Dr. Roland Kuhn and the MMCD we continued our efforts, without success, to establish a laboratory colony of *A. vexans* from the metropolitan area. Large numbers of eggs of *A. vexans* were collected from two different sites in Minnesota (Dakota county and Bird Island) and are being stored at 4 ° C until they are needed. These eggs will be hatched and larvae used in bioassays to determine the susceptibility of Minnesota larvae to the microsporidia we obtain for testing. These larvae will also be screened for indigenous strains of microsporidia.

We obtained from the U.S. Department of Agriculture (Insects Affecting Man and Animals Laboratory, Gainesville, Florida) a strain of the mosquito pathogenic *Nosema algerae*. Work is underway to develop the technology for the propagation of this pathogen in vitro and in insects. We will adapt the procedure developed by the USDA for the large scale production of this organism in caterpillars. We continued to work on the development of cell culture technology for cultivation and strain selection of entomopathogenic microsporidia. Improvements were made in the agarose cell culture method for isolating clones of microsporidia. These included procedures for improving attachment and immobilization of microsporidian infected cells in medium solidified with agarose. These findings will provide important technological information in our efforts to isolate clones of *N. algerae*.

*Edhazardia aedis*, a newly discovered microsporidian having considerable potential for the biological control of Aedes mosquitoes, was also obtained from the USDA in Florida. This microorganism survives desiccation well and is transovarially transmitted. Thus it has two features important to biological control. The ability of this pathogen to infect *A. vexans* has not been evaluated and we shall be evaluating this potential in the next few months. The ability of the larval infective haploid stage to replicate in mosquito and lepidopteran cell culture will also be evaluated. This pathogen is currently being propagated in larvae of the yellow fever mosquito, *A. aegypti,* but we need to develop protocols for its mass propagation and for evaluating its potency for *A. vexans.*

**Period 2: 1/92-7/92:** The strain of *Nosema algerae* we obtained from the USDA was characterized. In nature, this microsporidian infects only mosquito larvae and produces relatively few spores (1 million) per larva. Before it can practically be utilized as a biological control agent, we need to develop methods to produce large numbers of spores. To improve spore yields we



propagated the pathogen in corn earworm caterpillars (*Helicoverpa zea*). The yield in caterpillars was one thousand times higher than that in mosquito larvae. Studies are being done to evaluate the infectivity and virulence of these caterpillar-produced spores for larvae of *Aedes vexans* . The growth and development of *N. algerae* in tissue culture was also characterized. Spores produced in caterpillars were infective for cultured mosquito cells. *Nosema algerae* replicated and formed spores in cell lines isolated from embryos of a common Minnesota mosquito (*A. vexans*) and a predacious mosquito (*Toxorhynchites amboinensis*). In both cell lines cellular immune responses to the microsporidian were noted; these included lysosomal activation and melanin formation. These results provide us with important tools for strain selection to improve the efficacy of this microsporidian and analysis of the cellular immune responses. The growth and development of cultured *N. algerae* is being compared with the development of the parasite in the mosquito. The mosquito infectivity and virulence of pathogens produced in culture will be evaluated during the next period. Infected mosquito larvae and adults and cell cultures will be utilized in the immunological studies outlined n Objective II.

*Nosema furnacalis*, a microsporidian that normally infects only corn borers, failed to replicate in cells of *A. vexans*. This pathogen, which we are developing as a biological control agent of corn borers, is fairly specific for caterpillars and not likely to be useful for the biological control of mosquitoes.

*Edhazardia aedis* was found fraught with many shortcomings for utilization as a biological control agent of Minnesota mosquitoes. The microbe was difficult to propagate in the laboratory. The spores were temperature sensitive and could not be stored at temperatures below 40 F. The spores also had a short shelf life and quickly lost viability; we could not store aqueous suspensions of spores for more than a few weeks. Trials done by other researchers (Dr. Theodore Andreadis, personal communication) also indicate that it is not efficiently transmitted transovarially by mosquitoes in temperate regions. In the previous period we reported that *A. vexans* is suscepible to infection by *E. aedis* , nevertheless, these shortcomings must be overcome before we can utilize it for the biologiocal control of mosquitoes in Minnesota.

**Period 3: 6/92-1/93:** We continued to characterize species and strains of microsporidia identified to have potential for control of mosquitoes in Minnesota. These organisms are not easily propagated in the laboratory and much of our work this period was directed to improving mass propagation protocols. *Edhazardia aedis*: We developed protocols for producing spores in transovarially infected larvae of *Aedes aegypti.* In contrast to a protocol developed by the USDA, higher doses (1,000 to 10,000 spores) fed to younger larvae (1 to 2 days old) were needed to produce infected adult females. These protocols will assist in the mass propgation of the *E. aedis* spores needed in future trials with *Aedes vexans. Nosema algerae*: We characterized the chromosomal DNA of *Nosema algerae.* Fourteen chromosomes having sizes ranging from 280 kbp to 2,200 kbp were detected. The total genome was approximately 9,400 kbp. The features allow us to distinuish *N. algerae* from other nosemal species. The protocol to mass propagate *N. algerae* in caterpillars (corn earworms) was refined. A dose of 10 thousand spores was needed to produce 10 million spores in each caterpillar.

**Period 4: 1/93-6/93:** We have identified *Nosema algerae* as having potential for control of mosquitoes in Minnesota. A mass propagation protocol for producing *N. algerae* in corn earworm caterpillars, *Helicoverpa zea*, was developed. Spores produced in the laboratory

survived freezing (-21°C) or cold storage (5°C) for several months with retention of infectivity. We have identified *Edhazardia aedis*: as being unsuitable for the control of mosquitoes in Minnesota. The main drawback is its sensitivity to cold; *E. aedis* survived only 1 day at 5°C. We continued our work to develop a system for the continuous in vitro cultivation of *N. algerae*. The mosquito cell lines TAE12 and AVE1 were infected with high doses of spores in an attempt to overcome host cell defense responses and their rapid growth rates. Cultures infected with *N. algerae* were maintained for 2 months but host cells eventually overgrew the parasite. Similar results were obtained when we used a lepidopteran cell line, HZAM1, as a substrate for the parasites. We need to isolate slower growing and permissive cell lines from mosquitoes. Serological tests were done to develop methods for distinguishing between *N. algerae*, which infects both mosquitoes and caterpillars, from other microsporidia known to be specific for Lepidoptera. A test using antibody coated latex beads proved useful as a diagnostic test for this purpose. The test revealed there is no antigenic similarity in spore coat antigens of three different microsporidia specific for caterpillars with *N. algerae*. Our results on the in vitro biology and DNA characteristics of *N. algerae* will be prepared for publication in a scientific journal. We plan to continue the studies to isolate clones or geographical isolates of *N. algerae* that are suitable for the biological control of mosquitoes. These findings, as well as those of the previous 18 months, provide basic groundwork for the biotechnological manipulation and implementation of microsporidia as biological control agents of mosquitoes in Minnesota. The results obtained will serve as preliminary information for future grant proposals to NIH, USDA or USAID.

A. 6. Benefits: Success in these experimental approaches will provide a useful framework for evaluation of microbial control agents for mosquitoes. Methods will be established for the growth and maintenance of microsporidia in mosquito cell culture. Variability among laboratory selected strains (clones), evaluated by biological and biochemical criteria, will identify appropriate candidate strains for more advanced studies, testing, and eventual field application.

## B. Integrate desirable microbial properties with desired effects on mosquito reproduction and physiology.

B. I. Narrative: The long term efficacy of any microbial control agent depends on its infectivity and virulence in the target organism. Highly virulent pathogens, such as *Bacillus thuringiensis*, generally act to disrupt physiological processes such as feeding and digestion via the action of toxins. Less virulent pathogens that produce non-lethal effects may be desirable, particularly if they reduce fecundity or, in the case of disease vectors, compromise disease transmission. Non-lethal effects have the advantage of potentially allowing the microbial control agent to persist in insect populations and recycle effectively in the environment. In target insects, *Nosema* infections interfere with hormone-regulated behaviors and physiological processes such as metamorphosis, thus reducing fecundity in infected insects. These pathologies may be attributed to the production of hormones by the pathogen (Fisher and Sanborn, 1962). Virulence can be modified, and even compromised, by physiological defense mechanisms innate to the insect. Thus, from the control point of view, it is desirable to understand the biological interaction of the candidate microbial agent with its target insect, and its influence on mosquito biting behavior and reproduction, both of which are hormone-regulated (Meola and Readio, 1988). This component of our research plan is often ignored by laboratories working on



biological control agents, and **an important strength of our program will be the integration of studies on the pathogen itself with its effects on the mosquito.**

B. 2. Procedures: As candidate microbial agents that have been evaluated for their lethality in cultured cells and in mosquitoes become available (see objective A), each will be tested for its impact on the reproductive physiology of the mosquito. Mosquitoes will be exposed to lethal and sublethal doses of candidate *Nosema algerae* clones that represent a range of biological properties. Sugar-fed adults will be evaluated for longevity, blood-feeding behavior, and mating success under appropriate environmental conditions of temperature, photoperiod, and humidity. Fecundity, based on the number of eggs deposited and their viability, will be determined.

Continued development of *N. algerae* in adult tissues, and host defense reactions, will be followed microscopically. These analyses should allow us to choose from the various *Nosema* strains that will arise from Objective A, those that are of particular interest either from the control standpoint, or because their biology promises to provide new insights that will allow us to further refine this research program. The effect of the microorganism on mosquito physiology will be specifically evaluated by measuring two hormone-induced processes: 1) accumulation of ribosomes (eg. protein synthetic machinery) in the fat body, and 2) synthesis of the egg yolk protein. These two processes are essential for egg production in mosquitoes (Hagedorn, 1985; Hotchkin and Fallon, 1987), and molecular probes and appropriate biochemical techniques are already available for execution of these studies (Durbin et al., 1988).

Recent studies (Fallon, unpublished) indicate that synthesis of ribosomes in mosquito fat body is essential to both reproductive success and longevity. Moreover, it has been established that hormones from the mosquito brain, gut, and ovaries affect ribosome synthesis (Hagedorn, 1985). Ribosomes contain 90% of the total RNA in mosquitoes, and accumulation of ribosomes in fat body and in ovaries can be measured by extracting RNA using perchloric acid precipitationfollowed by alkaline hydrolysis (Hagedorn et al., 1973). Microsporidial infections that disrupt ribosome accumulation in key tisues would be expected to have an overall detrimental effect on survival, longevity, and fecundity. The specific nature of microsporidial effects on mosquito reproduction may provide insignts into whether the microsporidia themselves produce hormones, as was suggested by Fisher and Sanborn (1962). This potentially important early observation has never been followed up. If confirmed, hormone production by microsporidia may be a factor related to virulence that may be selectable in cell culture as described under section A above.

Ribosome accumulation will be further evaluated by measuring incorporation of tritiated uridine into ribosomal RNA (Hotchkin and Fallon, 1987), and expression of the egg yolk protein gene in infected mosquitoes, relative to control, uninfected mosquitoes will be monitored by polyacrylamide gel electrophoresis (Dhadialla and Raikhel, 1990). The sizes of the mosquito egg yolk proteins and their electrophoretic properties have been described in detail (see Hagedorn, 1985), and this information can readily be adapted to our system. The amount of egg yolk protein produced by infected tissues will be analyzed by densitometric scanning of gels to which quantitative amounts of protein have been added. Data will be correlated results from microscopic examination of the ovaries and successful hatching of eggs. These analyses will provide direct information on the effect of microsporidia on mosquito fat body and ovaries, key tissues whose biosynthetic activities are essential for both longevity and fecundity. Candidate *Nosema* strains

that show potential for interference with these processes will be desirable, and their properties will be reevaluated in mosquito cell culture, with **emphasis on defining a simple means of selecting additional strains with similar or enhanced potential.** Tissues from infected mosquitoes will be examined by microscopy to identify pathological changes associated with *Nosema* infection, and to correlate these pathologies with biochemical parameters that could easily be assayed in infected mosquitoes from the field, as a means of evaluating a control program.

B. 3 Budget

a. Amount budgeted: $75,000
b. Balance: $ 0

B. 4. Timeline for Products/Tasks

| | 7/91 | 1/92 | 6/92 | 1/93 | 6/93 |
|---|---|---|---|---|---|
| > Baseline values, fecundity & longevity | ********** | | | | |
| > Effects of candidate strains on fecundity, longevity | | ********** | ********** | | |
| > Effects on physiology, tissue pathology | | | ********** | ********** | |
| > Final report, manuscript preparation | | | | ********** | |

B. 5. Status: Period 1: 7/91-12/91. From the biological control point of view it is essential to understand the interaction of the candidate microbial agent with its target insect, and its influence on mosquito biting behavior, and reproduction, both of which are hormone-regulated. Examination of these components, often ignored by persons working with and/or applying biological control agents, contributes to our objective of integrating desirable microbial properties with desired effects on the target mosquito.

*Nosema* (and other microbial pathogens) are known to synchronize their developmental cycle with the physiology of the infected insect. This synchronization includes developmental responses to the hormonal cues initiated by blood feeding and associated with subsequent egg maturation. We have continued our efforts to understand the hormonal control of reproduction, with particular emphasis on the hormone produced by the ovaries. This hormone (20-hydroxyecdysone) is essential to egg development in mosquitoes. Since *Edhazardia aedis* is transmitted from one mosquito generation to the next via the eggs, it is likely that the life cycle of this microsporidian is synchronized to the egg maturation process, possibly using the same hormonal and biochemical cues. We have found that in ovariectomized mosquitoes, biosynthetic activity of the fat body is prolonged. This observation is supported by both biochemical and electron microscopy studies, and suggests one mechanism by which the ovaries, by means of an influence on the fat body, could contribute to the development and maintenance of a microsporidian pathogen.

In addition, the successful pathogen must escape the immune defense reaction in the mosquito, and replicate to sufficient levels to decrease mosquito fitness and viability. Mass production of microsporidia for biological control purposes must ensure the retention of these desirable properties, and at the same time maximize virulence to ensure effective control levels. Current efforts to define the physiological correlates of microsporidian virulence and the host immune response include the use of two dimensional gel electrophoresis to establish the profile



of approximately 1000 host proteins during the mosquito reproductive cycle. In ongoing studies, changes in this profile as a result of a microsporidian infection will be evaluated to 1) identify biochemical markers for microbial virulence and host immune response, and 2) identify properties of microsporidia that affect mosquito longevity and reproductive fitness. These results will be integrated into continuing efforts to develop *Nosema* and related microorganisms for mosquito control in Minnesota.

**Period 2: 1/92-6/92.** In this second budget period, we have extended our analysis of the immune response in mosquitoes, with the aim of developing assays that will be useful for evaluating the virulence of microsporidia in the laboratory and in the field. Although 4 major classes of immunity proteins have been described for large insects, such as silkmoths, next to nothing is known about mosquito immunity proteins, since these insects are small and contain limited amounts of blood. We have focussed particular attention on the lysozymes and cecropins, which act synergistically against pathogenic microorganisms such as bacteria and microsporidia.

Lysozymes are small (14,000 da) proteins that are typically expressed in both infected and noninfected insects. Lysozyme activity was first described in mosquitoes in 1990, in a study that identified activity specifically in adult salivary glands. We have adapted a procedure using agarose containing suspended Micrococcus lysodeikticus to assay total lysozyme activity in different stages of *Aedes aegypti*. Activity was most abundant in final instar larvae. During the next budget period, this assay will be extended to microsporidia-infected cells and larvae.

The second immunity activity that we have chosen to investigate is the cecropin protein, which is substantially smaller (3500- 4000 da) than lysozyme. Like lysozyme, cecropins have potent antimicrobial activity. Fortuitously, cecropin genes have already been cloned from the flies Drosophila and Sarcophaga, and we have obtained a cDNA clone to identify the corresponding mosquito gene. Once isolated, this gene will be used to monitor the tissue distribution, expression, and induction of cecropin activity in microsporidia-infected and control mosquitoes.

**Period 3: 7/92-12/92.** Efforts to characterize "immunity" proteins in mosquitoes continue. Using protein gel electrophoresis and radiolabelling techniques, we have identified a number of proteins whose synthesis is induced by exposure to microbes, and which may confer protective activity on the potentially infected mosquito. One of these proteins (a high molecular weight, secreted protein) is of particular interest because of its abundance, and because it serves as an indicator of microbial contamination of cell lines under lab conditions. The abundance of this protein is correlated with the dose of the microbial agent, and synthesis can be detected at 14 h after exposure. Efforts are in progress to define the time course of induction of this protein. The activity of this lysozyme degrades during frozen storage, and appears to be correlated with infectious dose of microsporidia.

Efforts also continue to obtain the antimicrobial cecropin gene, with the end goal of relating its expression to microsporidial infection. A genomic library has been constructed, and efforts to screen for the desired genes are in progress. In the course of this analysis, we have had to refine our assay technique for the detection of mosquito genes.

**Period 4: 1/93-6/93.** Characterization of the mosquito cecropin-like activity produced in infected mosquitoes and in a cell culture system indicate that the protein has a low molecular mass (less than 5000), is rich in basic amino acids, lacks methionine, and is stable under conditions of low pH electrophoresis. Conditions for purification of the protein have been established, including heat treatment, acid precipitation, and carboxymethyl cellulose chromatography. Funding for continuation of these studies will be sought from the NIH to support the microsequencing and chemical production of this protein at the U of M Microchemical facility. The protein will be tested for homology to a similar protein with activity against mosquito-borne human pathogens, and effects on microsporidia will be evaluated. These studies confirm a dynamic interaction between mosquito pathogens and the mosquito host that have potential influence on the efficacy of biological control agents. When such agents are released, these immunity functions will provide a means of monitoring success in controling the target insect, and thus on the effectiveness of the microbial agent in the environment. Moreover, these findings provide a basis for selecting, under laboratory conditions, potential microsporidian strains resistant to the mosquito immunity activity.

In addition to the cecropin activity that was characterized during this funding period, the research uncovered additional activities whose characterization remains to be undertaken. The nature of these activities with respect to microsporidia is at present unknown, but with future funding, will be investigated. These studies have increased our underatanding of parameters that affect the interaction of microsporidia with their mosquito host, and provide a basis for further evaluation of biological control agents and manipulation of their efficacy and selectivity in the environment.

B. 6. Benefits: Economically-successful microbial control agents of the future will be mass-produced in mosquito cell culture, most likely in fermenters. For effective implementation of these agents, it will be essential to evaluate maintenance and stability of desirable properties after multiple growth cycles. This study will provide information on the extent to which microbial agents selected for their pathogenicity in cell culture **behave as predicted in the organism.** Biochemical changes will be identified that can be used to evaluate control levels of these same agents during eventual use in the field. The outcome of this initial two year work period will be the establishment of a useful framework for evaluation of an effective microbial agent, setting the stage for eventual implementation of the agent in control programs.

## IV. Evaluation

Several criteria for evaluating the short and long term progress of this proposal are as follows. At the end of the1991-92 biennium, we expect to have 1) identified microsporidia with potential to control mosquitoes in Minnesota, 2) developed methods to grow and manipulate these pathogens under laboratory conditions, especially cell culture, 3) documented the variation in virulence of the pathogen and the feasibility of manipulating the determinants of virulence to improve strains of microsporidia for mosquito control, 4) biochemical and genetic markers, such as proteins or chromosomal DNA, will have been evaluated as a means of monitoring stability of the cloned Nosema strains, and 5) factors that contribute to pathogenicity of the agent will have been evaluated in the context of the physiology of the mosquito. **What we hope to accomplish in this initial phase of the research is to establish a generalizable protocol for the development and characterization of microbial agents that impact on mosquitoes.**



In the long term, the performance of these microorganisms under field conditions will need to be evaluated, requiring permission from the USDA, EPA, and APHIS and other regulatory agencies. Successful field application will also depend on development of cost-effective means of mass-producing the pathogen, either in insects or in cell culture. The markers that are identified in the course of characterizing the strains will facilitate field studies and allow monitoring of the persistence of the microbial agent.

Results of these studies will be incorporated into the dissertations of participating students, and will be submitted for publication in refereed journals. These results will also be used as a basis for seeking continued funding from Federal and State agencies.

## V. Context: Related current and previous work

A. **Need for additional studies:** While it is recognized that the microsporidia are significant suppressants of mosquito populations, their potential application to mosquito control has been limited. There is little history of strain improvement in the microsporidia, and there are no reports of efforts to genetically modify these microorganisms. Improving their potential for biological control will require a better understanding of their genetics. Our efforts to manipulate microsporidia in mosquito cell culture, and to evaluate variability among lab-selected strains, will begin to address this important issue.

B. **Supplementary aspects**: The ability to culture and manipulate these mosquito pathogens, and the technology to accomplish genetic studies and to evaluate strain improvement are already available. Thus, the basic approaches for this work are established. What will be new is their application to *N. algerae,* a specific pathogen of mosquitoes, and its effects on mosquito physiology. Studies on these specific problems have not been previously pursued, and will provide new scientific information.

C. **Related Past Accomplishments:** Past work on the culture of the microsporidia of lepidopteran insects has been funded by grants from the University of Minnesota Experiment Station to T. J. Kurtti. Several scientific publications resulted from this support, leading to the award of a new USDA grant to develop procedures for strain improvement of microsporidia for use in corn pest management. Past work on basic aspects of mosquito reproductive physiology has been carried out by A. M. Fallon at the University of Conn. and at Rutgers Medical School, with primary support from the NIH. LCMR funding through this award will facilitate the establishment of an active collaborative effort, combining the expertise from these two lines of study to address the issue of mosquito control in Minnesota. Although LCMR support for a two year period is being requested, it is likely that future LCMR proposals related to this project, or to other microbial agents of mosquitoes, will be submitted in the future.

D. Not applicable.

E. Biennial Budget System Program Title and Budget: Not Available at this Time.

## VI. **Qualifications**

1. Program Managers:

**Ann M. Fallon**
Associate Professor of Entomology
University of Minnesota

PhD: Biology, Queen's University, Kingston, Ontario, 1976
MS: Biology, Yale University, New Haven, Conn, 1974
BA: Biology, University of Connecticut, 1972

Dr. Fallon is author/coauthor on more than 30 refereed publications in the areas of insect biochemistry and molecular biology. She has pioneered in the development of techniques to investigate genetic regulatory processes in mosquitoes, and in cultured cells derived from mosquitoes, with long-term applications to mosquito control. She maintains an active research group, including 1 Assistant Scientist, 1 Postdoc, 3 PhD candidates, 2 MS candidates, and an Undergrad assistant. Her research is funded by competitive grants from the NIH and USDA.

Representative Publications:

Fallon, A. M. Optimization of gene transfer in cultured insect cells. J. Tissue Culture Methods, 12, 1-6, 1989.

Gerenday, A., Park, Y.-J., Lan, Q, and Fallon, A. M., Expression of a heat-inducible gene in transfected mosquito cells. Insect Biochem. 19, 679-686, 1989.

Park, Y.-J. and Fallon, A. M., Mosquito ribosomal RNA genes: Characterization of gene structure and evidence for changes in copy number during development. Insect Biochem. 20, 1-11, 1990.

**Timothy J. Kurtti**
Associate Professor of Entomology
University of Minnesota

PhD: Entomology, University of Minnesota, 1974

Dr. Kurtti is an insect microbiologist with experience in microbial control of pest insects and the transmission of human and animal pathogens by vector arthropods. His current research centers around the development of microsporidia for pest insect control and the transmission of the Lyme disease spirochete by the deer tick. His research support over the past10 years has been derived from the NIH, USDA, LCMR, WHO, AID, and private industry. Dr. Kurtti is the author or coauthor on more than 70 publications in insect pathology, medical entomology, and cell biology. He has broad experience with the microsporidia and related protozoa.



Representative Publications

Kurtti, T. J. and M. A. Brooks, 1977. The rate of development of a microsporidian in moth cell culture. J. Invertebr. Pathol. 29, 126-132.

Kurtti, T. J., K. R. Tsang, and M. A. Brooks, 1983. The spread of infection by the microsporidian, *Nosema disstriae* in insect cell lines. J. Protozool. 30, 652-657.

Kurtti, T. J. and U. G. Munderloh, 1986. Comparative studies on the infectivity of *Plasmodium berghei* gametocytes and ookinetes for gnotobiotic and xenobiotic *Anopheles stephensi.* J. Parasitol. 72, 706-710.

## VII. **Reporting Requirements**

Semiannual status reports will be submitted not later than January 1, 1992, July 1, 1992, January 1, 1993, and a final status report by June 30, 1993.

## VIII. **References cited in text**

Brooks, W. M., 1988. Entomogenous Protozoa. In: CRC Handbook of Natural Pesticides. V. Microbial Pesticides. Pp. 1-149.

Dhadialla, T. S. and Raikhel, A. S., 1990. Biosynthesis of mosquito vitellogenin. J. Biol. Chem., 265, 9924-9933.

Durbin, J. E., Swerdel, M. R. and Fallon, A. M.,1988. Identification of cDNAs corresponding to mosquito ribosomal protein genes. Biochim. Biophys. Acta, 950, 182-192.

Fisher, F. M., and Sanborn, R. C., 1962. Production of insect juvenile hormone by the microsporidian parasite *Nosema.* Nature 194, 1193.

Hagedorn, H. H., 1985. The role of ecdysteroids in reproduction. In Comprehensive Insect Physiology, Biochemistry, and Pharmacology (Edited by Kerkut, G. A. and Gilbert, L. I.), Vol. 8, pp. 205-262. Pergamon Press, New York.

Hagedorn, H. H., Fallon, A.M. and Laufer, H., 1973. Vitellogenin synthesis by the fat body of the mosquito *Aedes aegypti.* Develop. Biol., 31, 285-294.

Hotchkin, P. G. and Fallon, A. M., 1987. Ribosome metabolism during the developmental cycle of the mosquito, *Aedes aegypti.* Biochim. Biophys. Acta, 924, 352-359.

Kurtti, T. J. , Munderloh, U. G., and Noda, H, 1990. *Vairimorpha necatrix*: Infectivity for and development in a lepidopteran cell line. J. Invertebr. Pathol. 55, 61-68.

Klayman, D. L., Weeding out malaria. Natural History, Oct. 1989, pp18-27.

Meola, R., and Readio, J., 1988. Juvenile hormone regulation of biting behavior and egg development in mosquitoes. Adv. Dis. Vector Res. 5, 1-24.

Munderloh, U. G., Kurtti, T. J., and Ross, S. E., 1990. Electrophoretic characterization of chromosomal DNA from two microsporidia. J. Invert. Pathol. 56, 243-248.

Shemshedini, L. and Wilson, T. G., 1990. Resistance to juvenile hormone and an insect growth regulator in *Drosophila melanogaster* is associated with an altered cytosolic juvenile hormone binding protein. Proc. Natl. Acad. Sci. USA 87, 2072-2076.

**SHORT VERSION**
FOR THE PERIOD ENDING JUNE 30, 1993
Supported by the Legislative Commission on Minnesota Resources

Title: **Microbial/Genetic Strategies for Mosquito Control**
Program Managers: A. M. Fallon and T. J. Kurtti
Organization: University of Minnesota, Department of Entomology
Legal Citation: ML 1991, Ch. 254, Art. 1, Sec. 14, Subd. 9 (c)
Appropriation: $150,000
Balance: $0

**III. A.5**
**Period 4: 1/93-6/93:** We have identified *Nosema algerae* as having potential for control of mosquitoes in Minnesota. Spores produced in laboratory reared caterpillars survived freezing or cold storage for several months with retention of infectivity. *Edhazardia aedis*: was found unsuitable for the control of mosquitoes in Minnesota because of its sensitivity to cold. We continued our work to develop a system for the continuous in vitro cultivation of *N. algerae* in mosquito and lepidopteran cell cultures. A diagnostic test using antibody labeled latex beads was used to demonstrate antigenic differences between *N. algerae* and several other microsporidia parasites of Lepidoptera. These findings, as well as those of the previous 18 months, provide basic groundwork for the biotechnological manipulation and implementation of microsporidia as biological control agents of mosquitoes in Minnesota.

**III. B.5**
**Period 4: 1/93-6/93**. Characterization of the mosquito cecropin-like activity produced in infected mosquitoes and in a cell culture system indicate that the protein has a low molecular mass (less than 5000), is rich in basic amino acids, lacks methionine, and is stable under conditions of low pH electrophoresis. Conditions for purification of the protein have been established, including heat treatment, acid precipitation, and carboxymethyl cellulose chromatography. Funding for continuation of these studies will be sought from the NIH to support the microsequencing and chemical production of this protein at the U of M Microchemical facility. The protein will be tested for homology to a similar protein with activity against mosquito-borne human pathogens, and effects on microsporidia will be evaluated. These studies confirm a dynamic interaction between mosquito pathogens and the mosquito host that have potential influence on the efficacy of biological control agents. When such agents are released, these immunity functions will provide a means of monitoring success in controling the target insect, and thus on the effectiveness of the microbial agent in the environment. Moreover, these findings provide a basis for selecting, under laboratory conditions, potential microsporidian strains resistant to the mosquito immunity activity.

**1991 RESEARCH PROJECT ABSTRACT**
FOR THE PERIOD ENDING JUNE 30, 1993
Supported by the Legislative Commission on Minnesota Resources

Title: **Microbial/Genetic Strategies for Mosquito Control**
Program Managers: A. M. Fallon and T. J. Kurtti
Organization: University of Minnesota, Department of Entomology
Legal Citation: ML 1991, Ch. 254, Art. 1, Sec. 14, Subd. 9 (c)
Appropriation: $150,000

Statement of Objectives: To develop microbial agents that are environmentally safe and specific for mosquitoes, with emphasis on pathogenic microsporidia; to identify strains with efficient virulence; to integrate desired microbial properties with desired effects on mosquito reproduction and physiology. To establish preliminary data for continued funding for efforts related to biological control of mosquitoes in Minnesota.

Results: Strains of microsporidia including *Nosema algerae*, *Nosema furnacalis*, and *Edhazardia aedis* were evaluated as biological control agents of mosquitoes in Minnesota. *N. furnacalis* was not infective for mosquito cells; *N. algerae* replicated both in mosquito cell culture and in mosquitoes. Optimal yields of *N. algerae* for biological control can be produced in caterpillars (10 million spores per caterpillar), which have advantages of larger size and ease of handling, relative to mosquito larvae. *N. algerae* spores produced from caterpillars were infectious to mosquito cells, indicating the utility of this approach for mass rearing. *Edhazardia aedis* , which has attractive features such as the ability to recycle in the environment, was difficult to propagate in the laboratory, and spores were difficult to store in a viable state, limiting their potential for use as microbial control agents without additional research. The most critical factor limiting use of *E. aedis* in Minnesota is its marked sensitivity to cold. Important physiological processes that limit microsporidian infection in the mosquito were characterized. In particular, a small cecropin-like protein was produced by mosquitoes infected with microsporidia, and an in vitro model for characterizing this activity was developed. The activity had properties similar to the cecropin immunity protein previously described for the moth *Hyalophora cecropia* . Because the moth cecropin has activity against mosquito pathogens, such as the malaria parasite, and further research on this activity has potential implications for therapeutic control of mosquito-borne disease. In Minnesota, these diseases include LaCrosse encephalitis. In addition, these studies document the existence of defense reactions to microsporidia in mosquitoes, which must be factored into efforts to exploit these microbial agents in biological control strategies.

Project Results Use and Dissemination: A poster was presented by T. J. Kurtti and A. M. Fallon at the 1992 meeting of the Minnesota Academy of Sciences, at the Minnesota Science Museum, and at a College of Agriculture Retreat. A manuscript describing, "A cecropin-like activity from mosquito cells" by V. Hernandez. A. Gerenday, and A.M. Fallon is in preparation for the American Journal of Tropical Medicine and Hygiene (anticipated completion 9/93), and a poster describing these results was presented at the 1993 North Central Branch of the Entomological Society of America meeting, in Fargo, ND (March, 1993) by Vida Hernandez.