PROPOSAL FOR THE FY2004 IFA RESEARCH COMPETITIVE GRANTS PROGRAM

Texas Imported Fire Ant Research and Management Project

Title of project: Development of *Thelohania solenopsae* as a management tool for red imported fire ant through augmentation.

Lead principal investigators, contact information and signatures:

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Abstract

The protozoan pathogen *Thelohania solenopsae* has now been identified from red imported fire ant populations across the state of Texas. It is also known to occur in Louisiana and Oklahoma and detailed surveys are in progress. Where this pathogen is endemic in South America, it has a detrimental effect on the population density of the fire ants. On the average in South America, 6-11% of *Solenopsis* sp. mounds are affected in approximately 25% of the locations sampled, while 31% of the mounds examined in 80% of the infested counties in Texas were positive for the protozoan. Although this parasite density is not enough to completely eliminate the populations, the pathogen achieves these densities without human manipulation.

We propose to continue research that will lead to the development of three forms of manipulation that should prove successful. [1] is to identify factors affecting the natural increase of *Thelohania* in its current range. Understanding limitations on the geographic range and the effects of factors such as different strains of the pathogen will increase the chance of success in augmenting *T. solenopsae* to reduce the overall abundance of the red imported fire ant in the environment. [2] is to understand the environmental conditions that may trigger or stimulate the onset of the disease as well as the pathology of the disease in the host ants. [3] is to augment *Thelohania* directly in areas of economic interest, such as pastures, lawns and gardens. This will require the ability to mass rear spores and develop an infective spore bait. A bait containing spores would allow treatment of pesticide sensitive areas, such as parks and wildlife refuges. Once this technology is obtained, a sustainable infective and naturally spread biological pesticide will be available for home, agricultural and wildlife refuge use.

In 1998 the fire ant parasitic protozoan *Thelohania solenopsae* was found in populations of red imported fire ant, *Solenopsis invicta*, infesting Thorndale Texas. Samples of *S. invicta* from other locations throughout the southern United States also contained infected ants. This pathogen is known to occur in South America from Argentina to Brazil, but the strain in the United States is genetically different. In South America, *T. solenopsae* infects approximately a quarter of the sampled sites where black imported fire ants, *S. richteri* occur and between 6 and 11% of the mounds of *Solenopsis* sp. ants are infected. There is no doubt that disease initiated by this pathogen in ant populations is detrimental and that colonies are rendered unfit and die out. The effect of the pathogen on ant populations in the United States is presumed to be similar.

T. solenopsae is the first pathogen in Texas that can be sustained in fire ant populations and previous research funded by the Texas fire ant program has demonstrated it to be widely distributed in Texas. However, it is spread patchily, even in counties where it occurs. The survey was not designed to obtained detailed data on the prevalence of disease and the data are still under analysis, but indications are that natural infestations of the disease will likely be higher than the levels found in South America. While this will certainly affect the ant populations statewide, this level of infestation still falls below expectations in terms of managing ants to sub-economic levels or removing them entirely from the landscape.

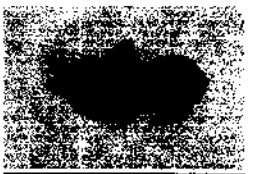
T. solenopsae distribution in Texas. Green counties indicate the protozoan is present in samples, blue counties indicate the protozoan was absent in samples, red counties were not sampled.

One means of converting *T. solenopsae* into a useful tool for fire ant control is to develop it into a packaged insecticide that can be used to augment natural infestations and to treat mounds in sensitive urban situations and home gardens. As a living organism, the protozoan must be treated differently than hormone baits or insecticides and its ability to affect management may vary. The epidemiology of the disease it causes is still not completely understood, but while this is being elucidated, the research and development required to formulate *T. solenopsae* into a viable insecticide may be completed.

Precedent for such an effort exists. The protozoan pathogen *Nosema locustae* has been developed into a bait (NOLO^{TM}) for grasshopper management. Although not noted for high efficacy against grasshoppers, many of the difficulties that diminish its utility are not present in *T. solenopsae / S. invicta* epidemiology. An analysis of its use in Africa by Lockwood et al. (1999) indicated that the erratic population levels of Acridid grasshoppers, the large area being treated (rangeland), the management of native pests in their native environment and finally the sophistication required of a user of biocontrol agents combined to defeat the efficient use of this pathogen. In the current instance, fire ants are invading pests, which contributes to large, constantly present populations. The goal of using an augmentative release in this instance is to saturate high value areas, at least initially. Such situations include but are not limited to locations where fire ants are invading environmentally sensitive areas, as in shorelines or parks and areas where pesticides cannot be applied directly, as in gardens or organic farms.

As do all obligate pathogens, *T solenopsae* also requires living cells to infect and grow. This would seem a large hurdle to leap when trying to do large scale production, but technology for the commercial scale has been developed as evidenced by the NOLO bait and may be adapted for production of *T. solenopsae*. In preliminary experiments, *T. solenopsae* cultures were maintained for 7 weeks in heterologous *Sf9* armyworm cells (*Spodoptera frugiperda*, #CRL-1711, ATCC, Manassas, VA) using Grace's Supplemented Insect Medium (Gibco) with media additives. Parasites appeared to reproduce slowly and large numbers of spores have not yet been produced in culture supernatants. Additionally, in preliminary experiments, *T. solenopsae* has been shown to infect 2 additional insect cell lines from mosquito larvae (*Aedes albopictus* C6/36, ATCC CRL-8910) and from silkworms (*Bombyx mori* BM-N, ATCC CRL-1660).

Trichrome stained *T. solenopsae* spores in *Sf9* cells, cytospin prep from culture



Calcolfuor MR2 stained spores in *Sf9* cell culture

Because *S. invicta* is an invading species and because it would be the only species targeted by treatment with *T. solenopsae*, chances of success in destroying mounds are very good. Although not all details of the complex epidemiology are yet known, a considerable amount of information can be presented. To date, we have developed DNA technology and identified histological stains that are diagnostic for the presence of the protozoan in ants. Combined with the adaptation of a rapid and simple DNA archiving technology, these protocols allowed us to accomplish a large scale survey for the presence of *Thelohania* in Texas. Samples of ants, stained slides and parasite DNA have been archived for subsequent research. We now know that the disease appears to spread very slowly in some circumstance, as uninfected colonies near infected colonies have been free of infection over several years (Cook, T. personal communication). In other circumstances, disease exploded though populations in within the space of less than a year (data from Charles Barr). Monogyne colonies can be infected, but we rarely find monogyne infected colonies in the field. We also know that there are three spore types (Sokolova and Fuxa, 2001) and it is likely that only one type is infective. We also discovered that the spores are most common in the meconium of pupating larvae and that workers consume a lot of the meconium and feed this material to larvae and the queen. We suspect that the spores are also passed to the larvae and that both larvae and workers are needed to spread the disease (Oi et al, 2001). Workers can not consume spores directly as they are too big (see Glancey et al. 1981), but larvae can (Petralia and Vinson 1978). We believe that by directing a bait to larvae we could increase the rate and level of infection. Further, since larval production is cyclic, the disease may be cyclic and the spores are primarily spread within a colony and not between colonies. All of this information suggests that the infection does not readily move from one colony to another in many circumstances and may not move easily to other workers within a colony.

Research into the development of the disease in individual ants and the discovery of a source and movement of spores within the nest has guided the hypothesis that development of a bait containing spores is feasible. Such a bait would allow us to do several things that would greatly increase the incidence and impact of the disease. By targeting the bait to the queen and larvae we could circumvent the cycle of meconium production and, in effect, have spore available to infect the larvae and queen at any time. We would be able to greatly increase the number of spores in a colony and thus increase the possibility of infecting more of the colony. We could also introduce the infection to monogyne colonies and introduce the disease to areas where it has not yet reached or where it either no longer occurs, it appears to die out in some areas after the infection has run its course (Vinson and Johnny Chen, unpublished information). Preliminary data indicating *in vitro* growth of *Thelohania* in a heterologous insect cell line is an encouraging step towards developing mass rearing technology needed for the development of a bait. The stage has been set to build on this progress and ultimately develop and disseminate a management program using augmentative releases of *Thelohania* as the means to control *S. invicta*.

Specific Goals and Objective of the program are:

- Development of PCR test to differentiate mono/polygyne fire ants, compare with head capsule widths from voucher specimens. (Mitchell, Snowden, Fuxa et al.). Archived ants and DNA samples are available from the completed survey for morphologic measurements and DVNA analysis. Sequences for the alleles of the *gp-9* gene have been published and will be used for development of primer sets.
- Determine if mono/polygyne presence affects *Thelohania* epidemiology (Mitchell, Fuxa et al.). Data generated in the first objective will also be used to analyze the relationship between the distribution of *Thelohania* in multiple or single queen colonies. Very few further samples will be needed.
- Development of an in-vitro parasite culture protocol (Consoli, Snowden et al.). Preliminary in vitro culture of *Thelohania* in a heterologous cell line is encouraging. Various insect cell lines, media composition and culture conditions will be evaluated for optimal growth and production of *Thelohania* spores in a tissue culture system. Such techniques have been used to develop a bait for the management of Acridid grasshoppers. Subsequently, the in vitro technique can be scaled up for mass rearing of infective parasite spores.
- Isolation and purification of the different spore types and evaluation of their infectivity as a precursor to development of a bait (Vinson, Snowden et al.).
- Routes of spore ingestion and the development of an effective bait (Vinson et al.) This issue is at the heart of an effective augmentation strategy. Ants may be able to avoid infection in some circumstances and the development of a bait that will ensure ingestion of spores is essential.
- Test the hypothesis of transovarial transmission by examination of eggs and ovaries for *T. solenopsae* stages by several techniques: fluorescent probes (DAPI/Rhodamine), PCR, in situ hybridization, and electron microscopy. (Fuxa et al.).

Expected outcome/Products/Management Tools or Approaches.

Knowledge that *T. solenopsae* is widespread in Texas indicates that it is or will become endemic in many areas of the state. However, to best use this disease agent to reduce RIFA populations, management strategies must be developed that enhance ant colony infection. The best means are the augmentation strategies. By further detailed analyses of samples and information obtained in the last cycle, we expect to develop recommendations for augmenting *T. solenopsae*. Success may in part be predicated on the climate, the structure of the colonies, the presence of strains of *T. solenopsae* and the presence of alternative hosts. Relying on the innate ability of *T. solenopsae* to spread naturally is inadequate for the purposes of this program. Understanding how the protozoan causes disease in ants, how the ant avoids infection, how environmental variables may stress ants and predispose them for infection will allow better understanding of the best times to release or transplant spores to ensure maximum suppression of colonies. Developing the technology to mass rear spores, incorporate them into a bait, and release them in areas of high ant density will allow for the long term weakening of the IFA population in a treated area. Further, the infection will proceed on a much more rapid basis and, on a smaller scale such as homeowner properties or

business properties within incorporated areas, this can be very important. Although the funding has been severely depleted by deep budget cuts, we anticipate being able to make good progress the targeted research areas by the end of the year. Should additional funding become available the following year, research should be completed.

Time line (for work to be done and anticipated technology transfer/implementation)
Many of these objectives will proceed simultaneously. It is therefore difficult to make an exact prediction. Early achievement of some of the goals, such as developing a PCR assay for mono/polygyne colonies, developing cell lines that produce an excess of spores, development of techniques for the purification of spores, and developing an acceptable bait, would accelerate the accomplishment of our goals. Very good progress will be made within the one year funding period. If further funding is forthcoming (from any source) the majority of the work can be completed in the first three fourths of the biennium and we will be in a position to begin to develop and evaluate recommendations for management implementation.

Relevance to the Texas Imported Fire Ant Research and Management Plan
Coordinated, Aggressive Research Program in Sustainable Solutions category. This support area states "Conduct fundamental studies on the physical and biological factors that limit fire ant development, including biological control, genetics/molecular biology, environmental restraints, and competition and re-invasion".

ANNUAL BUDGETS AND JUSTIFICATION FOR EACH PI

Mitchell, Forrest L.

A. Personnel	
Principal Investigator	
Graduate Students	
Undergraduate Students	$8,030
Secretarial – Clerical	
B. Capital Equipment	
C. Travel	
Domestic	
Foreign	
D. Other Direct Costs	
Materials and Supplies	$7,500
Publications Costs	$1,500
Chemical Analyses	$1,000
Services	$1,000

Budget Justification:
Personnel: Funds are requested for student workers
Supplies and materials are requested for the PCR and restriction enzyme analyses.
Chemical analyses and services are for sequencing reactions and the services are for purchase of DNA oligonucleotides as PCR primers.

ANNUAL BUDGETS AND JUSTIFICATION FOR EACH PI

Snowden, Karen F.

A. Personnel		
(1)	Research Associates K. Logan (10% time)	$3,900
	Post-Doctoral Fellows	
	Graduate Students	
	Undergraduate Students	
	Secretarial - Clerical	
B. Capital Equipment		$5,000
C. Travel		
	Domestic	
	Foreign	
D. Other Direct Costs		
	Materials and Supplies	$5,300
	Publications Costs	$1,120
	Chemical Analyses	
	Services	

Budget Justification

PERSONNEL:

Salary is requested for 10% effort for Kathleen Logan, Laboratory Technical Coordinator, in the Snowden research laboratory. Ms. Logan has more than 20 year of technical experience in immunology and parasitology research laboratories. She has worked with mammalian and avian microsporidial parasites for 7 years. Specific skills relevant to this project include the establishment and in vitro culture of microsporidia, where she has established 8 new isolates of the parasite, *Encephalitozoon cuniculi* from dog tissues (Snowden, Logan and Didier, 1999) and 1 new isolate of *Encephalitozoon hellem* from bird samples (the only existing avian isolate of microsporidia) (Snowden, Logan and Phalen, 2000). Additionally Ms. Logan has developed new methodology for testing and archiving DNA from infected fire ants (Snowden, Logan and Vinson, 2002). Ms. Logan will serve as the primary technical person to conduct in vitro culture experiments. She will evaluate various insect cell lines, media and media additives for their ability to support *Thelohania solenopsae* infection and growth in tissue culture.

SUPPLIES: A large number of disposable sterile supplies (pipettes, tubes, plastic culture flasks, sterile filters for media, gloves, etc), various media and media additives, fetal calf

serum, and purchased insect cell lines (American Type Culture Collection, Manassas, VA and other sources) are needed for tissue culture experiments.

EQUIPMENT: A minus 80 freezer for sample storage is essential to the project.

PUBLICATION COSTS: It is important to publish scientific manuscripts detailing data generated in this project. Funds are requested to offset publication costs such as journal page charges and production of photographic quality figures and tables.

ANNUAL BUDGETS AND JUSTIFICATION FOR EACH PI

Fuxa, J. R.

A. Personnel		
	Principal Investigator	
	Research Associates	
(1)	Post-Doctoral Fellows	$13,790
	Graduate Students	
	Undergraduate Students	
	Secretarial-Clerical	
B. Capital Equipment		
C. Travel		
	Domestic	
	Foreign	
D. Other Direct Costs		
	Materials and Supplies	
	Publications Costs	
	Chemical Analyses	
	Services	

Budget Justification

A post-doctoral specialist in microsporidiology is necessary to the project. This post-doc will focus on the life cycle of *Thelohania solenopsae*, including spore types, ultrastructure, detection techniques, and position ablative laser microbeam microscopy. The life cycle is complex and largely unknown at this time. Elucidation of the life cycle will contribute to almost every project objective relating to eventual field application: inducing infection by feeding (e.g., baits), detection of infection in ants, laboratory production of the microsporidium, and pathogen spread after release.

Consoli, Fernando.

A. Personnel		
	Principal Investigator	
1/2	Research Associate	$23,000
	Post-Doctoral Fellows	
	Graduate Students	
	Undergraduate Students	
1/8	Technical – Ant colony support	$4,000
B. Capital Equipment		
C. Travel		
	Domestic	
	Foreign	
D. Other Direct Costs		
	Materials and Supplies	$585
	Publications Costs	
	Chemical Analyses	
	Services	

Budget Justification

I am trained in the areas of insect in vitro rearing, the composition of insect diets, insect nutrition and biochemical physiology. I will be getting ½ of my salary as part of this project. I will continue working with Kathleen Logan (with Dr Snowden) on the development of in vitro mass production of *Thelohania* spores. Here my expertise in insect nutrition will be essential to this project. I will also be working with Mr. Hale who will be doing bioassays with spores to determine the route of infection and determining if spores remain infective when formulated in the different baits that will be developed by Mr Hale with my direction. As discovered by Dr Vinson and co-workers different foods are routed to the different fire ant stages (larvae, worker or queen) and this movement depends on the composition of the food and diet. Thus, we should expect that the infection of the fire ants should be determined by the type of spore and the route of the movement of spores in the fire ant colony, which is dependent of the type of food. Further, spores may be viable when formulated in a one food formulation (bait), but not another. Thus, spore viability becomes a question and will require evaluation. I will be working with Dr. Snowden and her group to evaluate spore viability.

Vinson, S. B.

A. Personnel		
	Principal Investigator	
	Research Associates	
(1/2)	Post-Doctoral Fellow	23,000
	Graduate Students	
	Undergraduate Students	
	Secretarial-Clerical	
B. Capital Equipment		
C. Travel		
	Domestic	
	Foreign	
D. Other Direct Costs		
	Materials and Supplies	775
	Publications Costs	
	Chemical Analyses	
	Services	

Budget Justification

No resources are going directly to Dr. Vinson as his salary is covered by TAES + TAMU. However, he will be coordinating the research and will be directing the half time post doctorial fellow. This person will also be working with Dr Snowden and Dr Mitchell in research on determining which spore type will infect the fire ant and will be involved in testing various routes of infection. However this will depend on Dr. Snowden and her laboratory in developing methods to separate and purify the various spore types. Further, they will be involved in continuing to supply the various types of spores. We plan to be in a position to begin to develop baits with infective spores by the end of this funding period.

SUMMARY BUDGET

Mitchell		19,030
Snowden		15,320
Fuxa		13,790
Consoli		27,585
Vinson		24,275