PROPOSAL FOR COMPETITIVE GRANTS PROGRAM: FY2003-2004

Texas Imported Fire Ant Research and Management Project

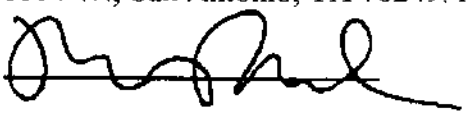
Title of project:

Isolation, identification and synthesis of IFA pheromones with management potential

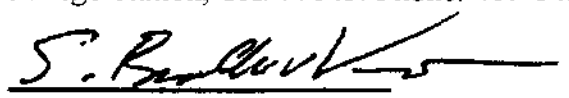
Lead principal investigators, contact information and signatures:

Richard J. Deslippe, Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409-3131. Phone: 806-742-2690; E-mail: Richard.Deslippe@ttu.edu

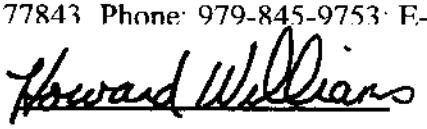
Robert Renthal, Department of Biology, The University of Texas at San Antonio, 6900 N. Loop 1604 W., San Antonio, TX 78249. Phone: 210-458-5452; E-mail: RRenthal@UTSA.edu



S. Bradleigh Vinson, Department of Entomology, Texas Agricultural Experiment Station, College Station, TX. 77843. Phone: 409-845-9754; E-mail: bvinson@neo.tamu.edu



Howard J. Williams, Department of Chemistry, Texas A&M University, College Station, TX. 77843. Phone: 979-845-9753; E-mail: williams@cbnmr.chem.tamu.edu



Abstract

Pheromones enable imported fire ant (IFA) colonies to prosper, and as their exploitation offers great management potential, we have developed a research program that targets IFA pheromones. Our coordinated efforts have resulted in several breakthroughs including (1) successful extraction and partial characterization of a queen pheromone that induces workers to eliminate sexual brood, (2) discovery of a putative pheromone-binding protein involved in mating, and (3) detection of both pheromones involved in mating and brood recognition, and a factor in the meconial fluid of queens that stimulates reproduction. Here we propose to continue work on the isolation, identification and synthesis of IFA pheromones. We will particularly target the compounds involved in development, mating, recognition and reproduction, as any alteration in their production, detection or timing of release would greatly reduce IFA infestation. A product composed of a complex of these pheromones would be especially effective, and its development is our priority and ultimate goal. We have related grant proposals pending (e.g., NIH and ATP) and will continue to pursue leveraging of Fire Ant Project Funding at both the state and federal levels.

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Background Information

Exploitation of the pheromone system offers great promise to manage *Solenopsis invicta*, and as a result, the chemical communication system of this ant has been particularly well studied. Some pheromones like the alarm compounds, cuticular hydrocarbons and recruitment chemicals have been identified and synthesized, but no pheromone regulating development, mating and reproduction has been characterized (Vander Meer et al. 1998). Knowledge of these latter compounds is highly desired, but identifying them is a major challenge in part because they are produced in minute quantities, they have complex structures, and/or the responses they induce are not immediate. To meet the challenge, we have developed new bioassays and incorporated new techniques, and in the process have made several key discoveries including (1) successful extraction and partial characterization of a queen pheromone that induces workers to eliminate sexual brood, (2) discovery of a putative pheromone-binding protein involved in mating, and (3) detection of both pheromones involved in mating and brood recognition, and a factor in the meconial fluid of queens that stimulates reproduction. Here we propose to continue work on the isolation, identification and synthesis of IFA pheromones, focusing on the compounds with the greatest management potential.

Hypotheses/Objectives/Proposed Work/Methods and Materials

Objective #1:

To complete sequencing and prepare for the expression of the queen proteins that induces workers to kill sexual brood. [*Deslippe & Renthal*]

We successfully extracted a water-soluble pheromone that induces workers to kill sexual brood (Deslippe 2002, Klobuchar and Deslippe 2002). The pheromone is produced by dealated queens, it is likely stored in the poison sac, and is stable at room temperature, presumably due to the presence of venom alkaloids that are also stored in the poison sac (Fig. 1). As the pheromone is most likely a protein, we subsequently analyzed extracts of poison sacs of both winged queens (which do not induce killings) and wingless queens for proteins by polyacrylamide gel electrophoresis. A 17 kDa protein was common to both winged and wingless queens, and unlike the former, wingless queens also generated a 14 kDa protein and lesser amounts of smaller proteins (Fig. 2). Based on partial characterization of the amino acid sequences of each of the two main proteins, we have concluded that the compounds are unique, but related to the antigen *Soli* II stored in the venom sac of IFA workers (Renthal and Deslippe, unpubl.).

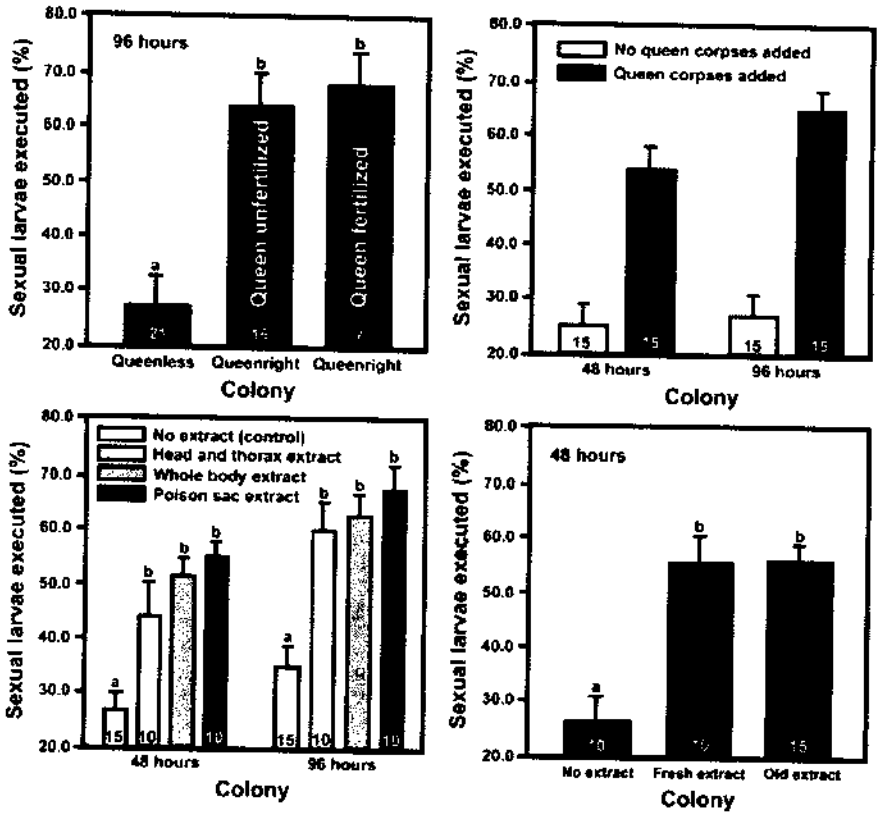

Fig. 1. **(a)** Sexual larvae killed by workers after 96 h in queenless and queenright colonies. Queens in queenright colonies were either fertilized or unfertilized. **(b)** Sexual larvae killed by workers in colonies either receiving or not receiving a queen corpse every 12 h. **(c)** Sexual larvae killed by workers in colonies receiving phosphate buffered solution (PBS) only (control), or receiving extracts of queen body parts in PBS. **(d)** Sexual larvae killed by workers after 48 h in colonies receiving PBS only (control), or receiving either fresh (1 day) or old (21 day) extracts of poison sacs of queens in PBS. Numbers within bars denote sample sizes, and shared letters above error bars designate means not differing significantly.

The findings suggest a precursor-product relationship whereby the smaller proteins are proteolysis products of the 17 kDa protein. They also raise the possibility that the proteins function both as pheromones and antibiotics. To address these issues, we will determine the full amino acid sequences of the 14 and 17 kDa proteins using both N-terminal Edman degradation and a Porton Instrument 2020 sequencer. The sequences will then be compared to those in

protein sequence databases, and used to prepare the corresponding cDNAs to be used as probes and for protein expression. We will insert the cDNA for the venom proteins in a vector and attempt to express the proteins in *E. coli*. Because these proteins are potentially cytotoxic, it may be necessary to use a modified host cell. If the project is extended to a second year, we would produce generous quantities of the purified proteins of interest and test them for activity in bioassays. These are the critical steps limiting the development of a product. While this scenario is ideal, if difficulties in cloning arise, we will use isoelectric focusing to separate large quantities of the proteins to enable testing via bioassays.

One bioassay would involve testing the hypothesis that one or more of the proteins induce workers to kill sexualized larvae. To do so, 60 subset colonies containing 20 sexual larvae in their final instars will be made from dozens of polygyne colonies of *S. invicta*. Control solutions of buffered saline and small volumes of both isolated proteins as well as a pooled extract of the proteins will be introduced into colonies every 12 hours. The number of dead sexual larvae after 48 and 96 hours will be counted and analyzed.

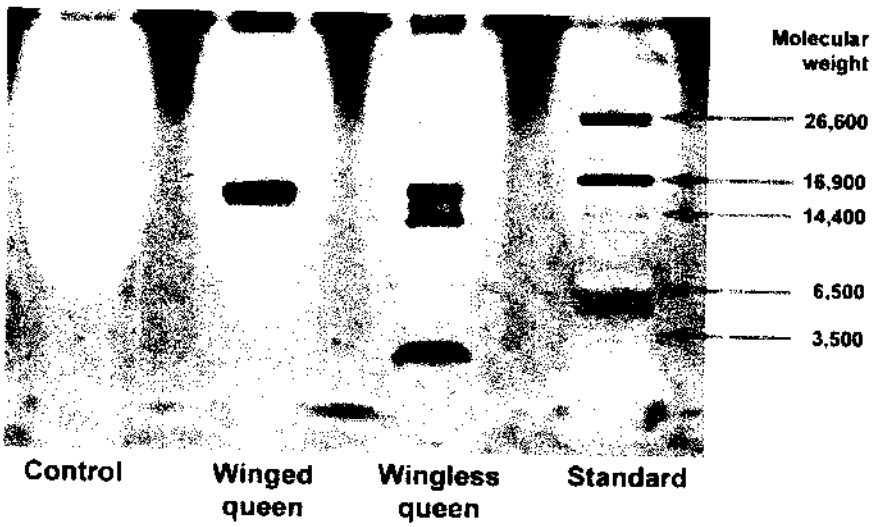

Fig. 2. Polyacrylamide gel showing protein bands of poison sac extracts of winged and wingless queens. Winged queens produced a 16.5 kDa protein whereas wingless queens produced a 16.5 kDa protein and substantial amounts of smaller sized proteins.

Although we strongly suspect that at least one of the proteins in the venom sac of queens functions as the pheromone of interest, our bioassays may rule out this possibility. If so, then in future experiments we will return to the queen extracts that induce workers to kill sexual larvae, fractionate them through a high performance liquid chromatography (HPLC) system and test the various fractions for activity. To identify the non-proteinaceous compounds, we will analyze the extracts by gas chromatography mass spectrometry (GC/MS) and HPLC/MS. To examine the extracts for additional proteins, we will conduct gel electrophoresis as we have done so repeatedly with the extracts of queen poison sacs.

Objective #2:

To purify and sequence the pheromone-binding proteins of male and worker antennae and identify their pheromone ligands. [***Renthal, Williams & Vinson***]

The antenna captures pheromones with pheromone-binding proteins. We have been pursuing a strategy of purifying IFA pheromone-binding proteins as a means of identifying pheromones that would otherwise be difficult to detect by conventional means. Most insect pheromone-binding proteins are members of the OBP/PBP family, the major low molecular weight proteins in most insect antennae examined to date. However, no OBP/PBPs have yet been identified in ant antennae. A fire ant OBP/PBP involved in queen recognition by workers, Gp-9, was recently reported in the thorax, but its mode of action is unknown (Kriger and Ross 2002). We previously determined the DNA sequence of the major antennal protein of fire ant males, and we identified it as apolipophorin-III (ALP-III) (Guntur et al. 2003). ALP-III was first found as a lipid transport protein in insect hemolymph (Narayanaswami and Ryan 2000), but many other functions have been discovered for it, including expression in a moth pheromone secretion gland (Liu et al. 2000). We also found that the major low molecular weight antennal protein of fire ant workers is similar to the sensory appendage protein (SAP) (Renthal and Velasquez, unpubl.). SAP is a member of an alternate odorant/pheromone-binding protein family, also known as chemosensory proteins (CSPs), and was recently reported in Argentine ant worker antenna and in the pheromone secretion gland (Jacquin-Joly et al. 2001).

We plan to extend our previous work to identify pheromones involved in mating and queen recognition. We must first clarify the role of ALP-III in the male fire ant antenna. We will express large quantities of this protein by cloning it in *E. coli*. Antibodies will be made to determine where it is expressed, using immunocytochemistry. Localization of ALP-III in the antennal sensilla (the pheromone-capturing hairs) would suggest a function in olfaction. The bacterial-expressed ALP-III would then be used to collect a pheromone ligand by affinity chromatography of extracts from winged females. The purified pheromone will be identified by GC/MS analysis. A similar approach will be used to identify the ligand carried by Gp-9, except that cloning is not necessary, as sufficient quantities of this protein can be isolated from the queen. We will also determine the DNA sequence of worker SAP and identify its ligands by affinity chromatography and GC/MS.

The isolated male fire ant ALP-III DNA will be ligated into a vector containing a transcription promoter for expression in *E. coli* (e.g., pET22). The vector will also incorporate a his-tag to facilitate purification of the expressed protein on a Ni column. The purified protein will be sent to a commercial lab (Pocono Rabbit Farm, Canadensis, PA) for polyclonal antibody production. If the project is extended to a second year, the antibody would be used to identify the location of ALP-III expression by immunocytochemistry, as follows. Male antennae would be dissected into subfragments in 4% formaldehyde, 0.1 M phosphate, pH 7.2, and soaked in this solution overnight at 4°C. After a buffer wash, the tissue would be transferred to a microscope slide, and each fragment will be cut with a scalpel into two halves, longitudinally. The tissue would then be briefly soaked in 0.1% Triton X-100 and washed with buffer containing 1% bovine serum albumen. This procedure would be followed by treatment with the rabbit polyclonal anti-ALP-III, a buffer-BSA wash, fluorescein-conjugated goat anti-rabbit IgG, and a final buffer wash. The stained tissue would be imaged in a BioRad MRC 1024 laser scanning confocal microscope. We have previously used this method to clearly observe actin in cells underlying sensilla in worker antennae, and it should provide a way to identify the location of expression of ALP-III in the male antenna.

Purified recombinant ALP-III will be immobilized on a Ni-NTA-sepharose 6B-CL column. Extracts of female alates or workers will be passed down the column. The extracting solvent will be pentane, and the column solvent will be an aqueous-organic mixture (e.g., 50% DMF in water). The column solvent will be selected after testing the conformational stability of ALP-III in various solvents, using circular dichroism spectroscopy. The protein-ligand complex will be directly eluted from the column with aqueous imidazole, re-extracted into pentane, and analyzed by GC/MS to identify bound pheromones.

The N-terminal protein sequence of the major worker antennal protein, which we previously determined, will be used to obtain the full DNA sequence of this protein. Antennal RNA will be isolated from about 1000 workers and reverse-transcribed into cDNA. PCR will be done on the antennal cDNA using degenerate 5' primers based on the N-terminal protein sequence and oligo dT as 3' primers. PCR products in the 400-500 nucleotide range will be isolated, ligated with pBluescript plasmid digested with the same enzyme, transformed, and the resulting colonies will be screened with PCR isolate probes. DNA sequences of plasmids isolated from transformants will be examined for the 5' sequence of the worker antennal protein. Those clones with the expected 5' sequence will have the full DNA sequence determined on an ABI 3100 Avant sequencer. The derived protein sequence will be confirmed by mass spectrometric analysis. Assuming that the sequence corresponds to a SAP/CSP family member, as expected, the same method outlined above for ALP-III can be used to identify a pheromone from worker and queen extracts, using affinity chromatography on a Ni-NTA column, if the project is extended to a second year.

Objective #3:

To identify the pheromones involved in brood recognition, mating and reproduction. [***Vinson, Williams & Deslippe***]

We have determined that virgin queens release volatiles just prior to and during mating flights, workers recognize and distinguish brood at least partly through chemical signals, and functional queens increase reproduction in response to a compound in the meconial fluid. These discoveries warrant further exploration as they involve key aspects of colony success and the responsible compounds could be developed into effective products that help reduce IFA infestations. Before products can be produced, the compounds must be identified, synthesized and further tested. Here we propose to pursue these intermediate steps for the pheromones involved in brood recognition, mating and reproduction.

To isolate active compounds involved in nuptial flights, we will conduct bioassay driven standard chemical extraction and use purification techniques, including solid phase microextraction. Microextraction adsorbs volatile compounds from the local atmosphere around an organism (Frerot et al. 1997). The compounds can then be introduced directly into capillary GC/MS. Further, no solvent is used in microextraction, and thus, the GC column can be monitored with electroantennographic detection system to confirm biological activity (Zhang et al. 1999; Baehrecke et al. 1989). We will purify and identify active compounds using GC/MS and nuclear magnetic resonance (NMR), and once the pheromone structure is deduced, we will design synthesis methods for the pheromones, if the project is extended a second year. The compounds will then be synthesized and their activity confirmed by bioassay. To identify the

reproductive stimuli in the meconium fluid, we will isolate the factor by HPLC and identify it with MS and/or NMR. The information will then be used to design an antagonist.

The same basic approach will be used to identify the pheromones involved in brood recognition. In this work, we will (1) extract compounds from each larval stage and pupae of *S. invicta*, (2) test the extracts for pheromonal activity in behavioral bioassays, and (3) identify the chemicals in the extracts and determine the ones responsible for social facilitation of eclosion and larval recognition. If the project is extended a second year, we will synthesize suspected pheromones and test both the synthesized and natural compounds for pheromonal activity. Little research has been conducted on brood pheromones of *S. invicta* over the past 20 years. In the 1970s and early 1980s, there was much interest in such pheromones, but the research was controversial, as the techniques that were used were simplistic. Chemical ecology, as a discipline, has matured considerably, and it has become more mechanistic and sophisticated. As a result, some doubt exists on the assignment of "brood pheromone" function to any semiochemical in IFA. It is important to note, however, that brood tending, social eclosion and retrieval behavior are readily observed and are uncontested. The uncertainty that remains involves the specific mechanisms by which these behaviors are achieved. Uncovering these mechanisms and the brood pheromones would be especially valuable from a management perspective. For one, they would enable a dramatic increase in the specificity of toxic baits and thereby target only *S. invicta*.

Expected Outcome/Products/Management Tools or Approaches

We expect to continue to increase our understanding of the communication system of IFAs, and use this knowledge to exploit the system. We have particularly targeted the compounds involved in development, mating, recognition and reproduction, as any alteration in their production, detection or timing of release would greatly reduce IFA infestations. A product composed of a complex of these pheromones would be especially effective, and its development is our priority and ultimate goal. For the peptide pheromones, we can isolate the relevant genes, and clone them into an expression vector, methods now being developed by the genetics group. The vectors could then be induced to produce large quantities of the pheromones.

Time Line

Objective #1: Protein pheromone of queens

	9/03	1/04	5/04	2004/05
Sequencing				
Cloning				
Expression				
Bioassays				

Objective #2: Pheromone-binding proteins

	9/03	1/04	5/04	2004/05
Sequencing				
Cloning				
Expression				
Identification				

Objective #3: Releaser pheromones and meconial factor

	9/03	1/04	5/04	2004/05
Bioassays				
Identification				
Synthesis				

Relevance to the Texas Imported Fire Ant Research and Management Plan

The main goal of research funding from the Management Plan is to create and improve technology for suppressing and controlling *S. invicta*. Our program directly addresses the goal, as it aims at producing new effective weapons to combat IFA infestations. The basic strategy is to understand and exploit the chemical signals involved in communication.

Outside/Leveraged Related Funding

Related grant proposals are currently pending (e.g., NIH and ATP), and we will continue to pursue leveraging of Fire Ant Project Funding at both the state and federal levels.

Literature Cited

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ANNUAL BUDGETS AND JUSTIFICATION FOR EACH PI

Deslippe, R.J.

A. Personnel	
(1) Principal Investigator	$7,900
(0) Research Associates	$0
(0) Post-Doctoral Fellows	$0
(2) Graduate Students	$13,700
(1) Undergraduate Students	$0
(0) Technician	$0
B. Capital Equipment	$0
C. Travel	
Domestic	$2,900
D. Other Direct Costs	
Materials and Supplies	$7,300
Publications Costs	$1,200
Chemical Analyses	$750

Budget Justification

Principal Investigator: Dr. Deslippe will be actively involved in the bioassays and chemical analyses, and will direct and oversee the graduate students. He will be the liaison between his lab and the labs of Dr. Renthal (for the protein related studies) and Drs. Vinson and Howard (for the synthesis of pheromones). The budgeted amount is for partial summer salary.

Graduate students: Two graduate students (Jilla HaghiPour [M.Ss.] and Issam Al Diri [M.Sc.] will be involved with the research fulltime. Jilla will continue to dissect poison sacs of queens, run polyacrylamide gels of proteins, conduct chemical analyses, and clone the cDNA of the pheromone that induces workers to kill sexual larvae. Issam will conduct bioassays and chemical analyses of larval and pupal extracts at TTU. If the project is extended a second year, he will also work with the lab members of Drs. Vinson and Howard on synthesizing promising pheromones and the testing of the compounds for biological activity. The budgeted amount is for partial salaries, and the remaining balance on the salaries will come from appointments at TTU as teaching assistants.

Undergraduate student: Amelia Haffner will be involved in the collecting and maintenance of fire ant colonies. She will also assist me and my graduate students with the set-up for bioassays. She is currently an HHMI fellow and the program pays her full salary (20 hours/week during fall and spring semesters, and 40 hours/week during the summer months.)

Travel: Funds are needed for me and my students to attend conferences and meetings with Drs. Vinson, Howard and Renthal. They are also needed for several collecting trips for fire ants, as many colonies with sexual larvae are needed, especially for bioassays.

Other Direct Costs: Many supplies are needed ranging from Criterion™ pre-cast gels, PVDF membranes for electroblotting, toner cartridges, food for ants, Fluon™, plastic- and glass-ware for experiments, solvents, and columns for GC and HPLC systems. Funds are also needed to publish several papers from our ongoing research with fire ants, and although I have an arrangement whereby I can run most chemical analyses at no charge, I will have to pay for amino acid sequencing on the Porton Instrument 2020 sequencer.

Renthal, R.

A. Personnel		
(1)	Principal Investigator	$0
(1)	Research Associate (75% time)	$25,500
(0)	Post-Doctoral Fellows	$0
(1)	Graduate Students	$0
(0)	Undergraduate Students	$0
(0)	Technician	$0
B. Capital Equipment		$0
C. Travel		
	Domestic	$0
D. Other Direct Costs		
	Materials and Supplies	$8,000
	Publications Costs	$250
	Chemical Analyses	$0

Budget Justification

Personnel: Research associate (Daniel Velasquez), 75% time; duties include maintaining ant colonies, dissecting ants, extracting proteins, running polyacrylamide gels of proteins, isolating RNA, and performing PCR.

Materials and Supplies: Costs include supplies for ant maintenance, electrophoresis supplies, N-terminal amino acid sequence analysis, PCR primers and enzymes, DNA sequence analysis

Vinson, S.B.

A. Personnel		
(1)	Principal Investigator	$0
(0)	Research Associates	$0
(1)	Post-Doctoral Fellows	$30,000
(0)	Graduate Students	$0
(0)	Undergraduate Students	$0
(0)	Technician	$0
B. Capital Equipment		$0
C. Travel		
	Domestic	$0
D. Other Direct Costs		
	Materials and Supplies	$3,750
	Publications Costs	$0
	Chemical Analyses	$0

Budget Justification

Dr. Vinson: No direct cost to the project. He will direct and oversee the bioassays, obtaining ant material and provide ant expertise. He will work closely with Dr. Williams, Deslippe and Renthal to insure coordination with elements of this project and, the Reproduction and Resistant Grass projects.

One Post-Doctoral Fellow: This person will be responsible for developing and conducting the bioassays to isolate the queen reproductive stimulant material and the worker ant attractant in the meconium, the antennal secretion and other lipid soluble substances that must be isolated from extracts. Some of these extracts will come from and be in collaboration with Dr. Deslippe. Once an active material is isolated, this person will work with Dr Williams to conduct the bioassays needed to purify, identify and synthesize the factor (s), or if the compounds are water soluble this person will work with Dr. Renthal.

Other Direct Costs: Supplies include the food used to feed the ants. We also use containers, gloves, and other supplies necessary to maintain ant experiments.

Williams, H.

A. Personnel		
(1)	Principal Investigator	$0
(0)	Research Associates	$0
(1)	Post-Doctoral Fellows	$27,000
(0)	Graduate Students	$0
(0)	Undergraduate Students	$0
(1/8)	Technician	$6,000
B. Capital Equipment		$0
C. Travel		
Domestic		$0
D. Other Direct Costs		
Materials and Supplies		$750
Publications Costs		$0
Chemical Analyses		$0

Budget Justification

Dr. Williams: No direct cost to the project. He will direct the bioassay driven separation, purification and identification of relevant compounds involved in the fire ants biology. He will synthesize identified compounds and work with students and post doctorial personnel to confirm compound activity. He will work closely with Dr. Vinson, Deslippe and Renthal to insure the identification of relevant compounds.

One Post-Doctoral Fellow: This person will be responsible for developing and conducting the bioassays to evaluate the functions of the volatile and lipid soluble types of pheromones such as those that will be isolated using the solid phase microextraction technique. Some of this research will be in collaboration with Dr. Deslippe and Dr. Renthal. This person will also work with Dr Williams to run the GC and other chemical separation equipment.

One eighth time ant maintenance person: This person (Mrs. Ellison) directs ant collection and evaluation. Evaluation is essential to insure that the ants collected are true fire ants and are not collected from pesticide treated areas that would compromise experiments. She determines if they are hybrids, monogyne, polygyne, diseased, or native. The ants are cataloged to location to recollect if needed. If *Thelohania* infected ants are needed, she knows where to go to get them.

Supplies: For chemicals that are used in isolation, purification and synthesis. Besides the chemicals, columns and other materials used in chemical separation must be often be replaced.

Chemical analyses: The mass spectrometer and NMR equipment needed to identify the purified chemical compounds is in another department. We must contract for the use of this equipment; however, I have access to this equipment and get a discount.

SUMMARY BUDGET
(Deslippe, Renthal, Vinson & Williams)

A. Personnel		
(4)	Principal Investigators	$7,900
(1)	Research Associates	$25,500
(2)	Post-Doctoral Fellows	$57,000
(2)	Graduate Students	$13,700
(0)	Undergraduate Students	$0
(1/8)	Technicians	$6,000
B. Capital Equipment		$0
C. Travel		
	Domestic	$2,900
D. Other Direct Costs		
	Materials and Supplies	$19,800
	Publications Costs	$1,450
	Chemical Analyses Services	$750

Budget Justification

The budgets for each PI are justified on the individual budget pages which came before this summary budget. The overall breakdown of the total budget for the 2004 fiscal year is as follows: 73.6% salaries, 0.0% capital equipment, 2.8% travel and 23.6% maintenance and operations.