PROPOSAL FOR THE FY2004 IFA RESEARCH COMPETITIVE GRANTS PROGRAM

Texas Imported Fire Ant Research and Management Project

Title of project: Developing the concept of reproductive control as a method to reduce the IFA population.

Lead principal investigators, contact information and signatures:

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Abstract

Minimizing or suppressing the reproductive potential of an organism is effective to eliminate its population. The proposed research will identify novel means to disrupt the reproductive cycle in IFA queens. We have discovered IFA queens contain two yolk proteins (vitellogenins). Vitellogenin-182 is present in both unmated (alate) and mated (queens) reproductive females. Vitellogenin-171 appears only in mated queens, suggesting that it may be essential to produce the profuse number of fertile eggs needed to form a fire ant colony. Presence of a vitellogenin receptor that binds and transports vitellogenin into the developing egg to form yolk may be the critical step for egg formation. We have cloned the cDNA for the genes of vitellogenin-182 and the vitellogenin receptor, and we are currently cloning the gene for vitellogenin-171. We have also identified some of the factors that control the reproductive potential of IFA. These include the important role that larvae have in digesting nutrient proteins needed for reproduction, but even more dramatic is the discovery that the meconium (produced by larvae at the time of pupation) greatly stimulates egg production. It is also known that the primary queens suppress the reproduction of subordinate queens, and this dominance hierarchy of reproduction in polygyne colonies provides an opportunity to conduct experiments that should lead to the isolation of the dominance-regulating factor(s). Our current genetic and behavioral information positions us to develop probes to accurately measure the biochemical steps involved in IFA reproduction. These probes will allow us to isolate and identify the factors that regulate IFA reproduction. Identification of the regulatory factors that control reproduction will provide us with the tools and strategies to control these factors and disrupt IFA reproduction.

Background information.

Insect reproduction involves the synthesis of one or more yolk precursor proteins (vitellogenins) by the fat body in response to juvenile hormone, 20-hydroxyecdysone or both, depending on the species (Hagedorn, 1980; Belles, 1995). Vitellogenins (Vg) are secreted into the blood and carried to the ovaries where they are taken up by the maturing eggs by a vitellogenin receptor (Valle, 1993). The Vg receptor is a cell-surface protein that binds the hemolymph-borne Vg, transports it into the egg and deposits it in the yolk to form vitellin, the mature yolk protein (Raikhel, 1987). The receptor is re-cycled back to the cell surface for another round of Vg binding. Ultimately, vitellin is degraded to provide amino acids for embryonic growth.

We have identified the basic sequence of physiological events related to reproduction in IFA queens, and it appears unique. Two VGs (mol. wt = 171 kDa and 182 kDa) are synthesized for egg formation in IFA queens (Lewis *et al.*, 2001; Lewis *et al.*, 2002)]. VG-182 is present at all times in adult queens, even before mating and egg laying occur, and VG-171 is present only after mating, when egg laying is initiated. In most insect species, the onset of Vg synthesis results in yolk formation and egg production. However, since VG-182 is present in unmated alate females without egg formation, we propose that the regulatory event for egg production in reproductive female IFA may be the synthesis of VG-171 or activation of the Vg receptor. We have cloned the genes for VG-182 (Lewis *et al.*, 2003) and the Vg receptor (Chen *et al.*, 2004). A gene fragment for VG-171 has been identified and the full gene is being cloned with help from the IFA Genetics program. The identification of these genes provides us with probes we can use to unravel the reproductive picture.

We found that reproduction is highest in monogyne queens. In polygyne colonies there is a dominance hierarchy, with the dominant queen having the highest level of reproduction; whereas, the lowest subordinate queen only lays a few eggs per week [Chen and Vinson, 2000; Cassill et al., submitted]. We know that both the dominance can change, and that the VG-171 content varies among the multiple queens in polygynous colonies. The different levels of reproductive capacity suggest that there are controls that result from the differences in queen dominance. We also find that reproduction is greatly increased when larvae pupate. We have identified the larval meconium (larval excrement at pupation) as the agent that increases queen reproductive capacity. The meconium is highly attractive to workers and the workers remove the liquid and feed it to the queen [Cassill and Vinson, manuscript in prep.].

However, it is unclear why there are two VGs in the fire ant with different periods of synthesis and how they are independently regulated. Also, the relationships are unclear between the receptor and the two VGs. Does the receptor bind both VGs? Further, when is the receptor protein produced? What hormone in the fire ant controls the presence/absence of the receptor? How is the synthesis and activity of the VGs and receptor(s) regulated? Are there yet to be discovered hormones involved? Is the difference in the blood of the two VGs in polygyne queens related to queen dominance? Are the differences in the relative amounts of the two VGs in the different types of eggs layed by queens related to the fate of the eggs? Does the meconium factor affect the VGs or the VG receptor? Now that we are in the position to complete the development of probes to these various molecules, we can answer these questions.

Since we now have, or will soon have, the cDNAs for the two VGs and the VG-receptor, we can develop specific probes to determine the levels produced, what the regulatory factors are, and how to turn of the events leading to reproduction. We will isolate and identify the reproductive stimulants and controls and conduct bioassays with the cooperation of chemists to identify the active regulators (see the Pheromone proposal –Dislippe et al).

Hypothesis/Objectives/Proposed Work/Methods and Materials.

Objective # 1. Develop a method to quantify the two vitellogenins (**Vinson, Pietrantonio**).

We will develop assays to differentiate VG-171 and Vg–182. Several approaches will be assessed to identify the most efficacious and simple method. Lectins derived from different sources have differential binding capacities for the various sugar moieties of glycoproteins depending on the source of the lectins. Vitellogenins are glyco-lipo-phopho-proteins, and VG-171 and –182 will be separated by SDS-polyacrylamide gel electrophoresis and exposed to lectins from several sources to determine if the two proteins show differential lectin binding. If differential binding is detected, it is possible to use the various lectins of interest bound to Dynabeads™ to bind each VG separately as a basis for VG separation and quantitation. We could also use 2-D electrophoresis to separate and quantitate the two VGs, alone or in combination with lectin binding. Finally, once the base sequences are defined for both VG genes (one is complete – Lewis, et al., 2003; the other is in progress and should be done in the next several months), we can compare the deduced amino acid sequences and identify unique differential sequences. The unique sequences can be used to synthesize peptides and a mixture of these unique peptides for each VG can be

used as an antigen to produce antibodies specific to each VG. The antibodies will be confirmed for specificity and bound to Dynabeads™ that can be used to isolate and quantitate each protein.

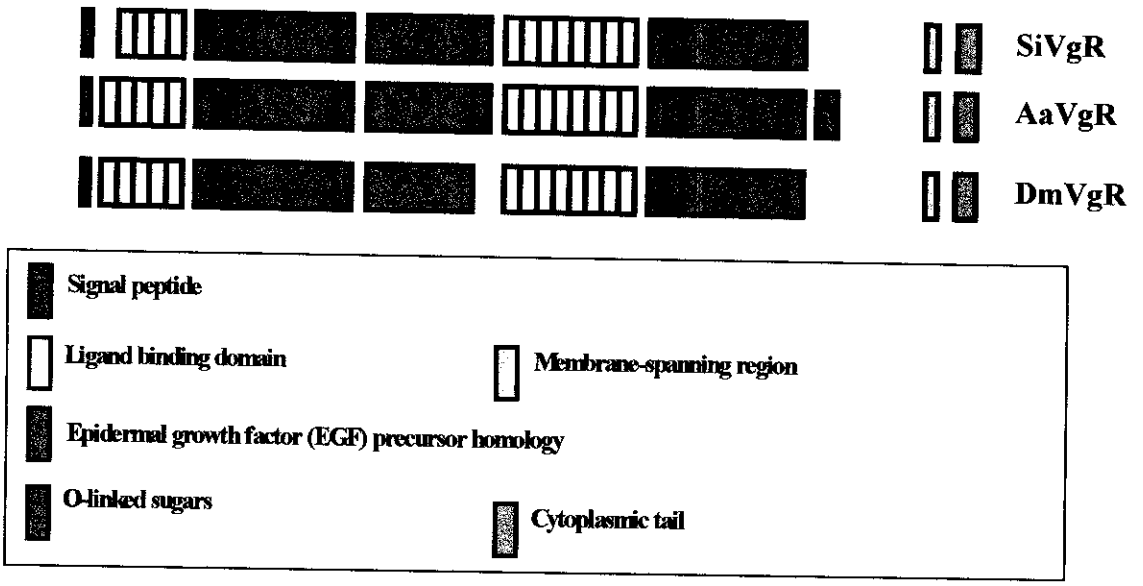
Quantitative PCR (semi-quantitative or real time PCR TaqMan) can be used to determine the presence and relative amounts of the transcripts (mRNAs) for VG-171 and -182 in the fat body of queens or the vitellogenin receptor in the ovaries. For the latter experiments with the receptor we have used as a positive control a fire ant filamin fragment that we cloned in the previous funding period (Pietrantonio et al., 2002).

Altogether these experiments will show the timing of expression for the genes. This information will demonstrate when, in the reproductive cycle, the regulatory hormones are released endogenously within the ant. Knowledge of when the genes are unstimulated, but sensitive to hormone stimulus, can be combined with *in vitro* exposure of the fat body (VG genes) or ovaries (VG receptor gene) to hormones or gland extracts to determine the hormones that stimulate specific gene expression.

We will focus on fat body expression of VG-171, in particular. Since VG-171 appears only after mating as reproduction is becoming maximally active, VG-171 regulation may be essential for colony founding, therefore, its disruption may provide a weak link to stop reproduction and prevent colony formation.

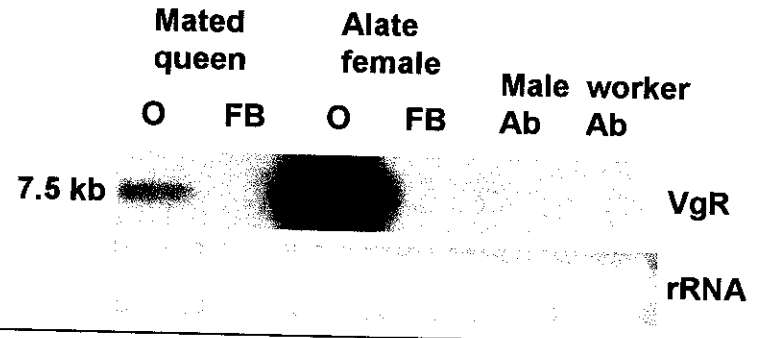
Objective # 2. Determine the temporal pattern of receptor synthesis in virgin queens (alates) and in mated queens up to 30 days after mating and receptor synthesis in response to insect hormones (***Pietrantonio, Vinson***)

We have cloned the full-length cDNA for the Vg receptor (Chen *et al.*, 2004).



The figure above demonstrates that the structural domains of the cloned IFA Vg receptor are highly similar to those for the other two known insect vitellogenin receptors for the mosquito *Aedes aegypti* (AaVgR) and the fruitfly *Drosophila melanogaster* (DmVgR).

We have determined by northern blot analysis that the receptor transcript (mRNA) expression level is higher in alate females (see figure).

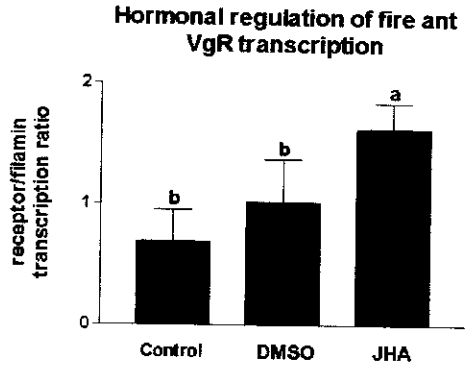


O= ovary; FB= Fat body; Ab= abdomen

VG receptor transcript expression is much lower in mated queens during active egg formation. Since the receptor protein is re-cycled during active uptake of VG for vitellogenesis, large amounts of new receptor do not need to be made, only small replacement amounts that require minimal gene expression.

As we earlier determined that VG-182 is present in the hemolymph at all times while VG-171 is found only in mated females, this suggests that egg production may be regulated by synthesis (presence) of this receptor protein and its activity to bind and carry Vg into the developing egg.

Preliminary results (see bar figure below) indicate that juvenile hormone (JH) stimulates receptor transcriptional (mRNA) expression, but JH has no effect on VG-182 synthesis. In the experiment shown below, the mRNA for fire ant filamin, a protein involved in ring canal formation in insect ovaries is used as internal control.



The **hypothesis** is that egg formation is hormonally controlled by the presence or absence of the active receptor in the ovaries of reproductive females. We will develop a specific polyclonal anti-peptide antibody against this receptor and we will be confirming receptor protein expression by western blot using this specific antibody.

We propose to determine these complex regulations and disrupt receptor activity using insect growth regulators or other available compounds to validate this receptor as a target for fire ant control. (Determining the binding and carrying capacity of this receptor for either Vg is beyond the scope of the present proposal but we plan to leverage funds for this idea).

Objective #3. Determine if the difference in egg production in polygynous colonies is due to vitellogenin synthesis, release, or uptake and determine the control for this difference. (***Vinson***).

It is established that reproductive queens suppress the reproduction of other queens in ant colonies, and efforts have been undertaken to identify the factors involved. However, these efforts have been hampered by a lack of a sensitive bioassay. Egg-laying has been used but is confounded by multiple behavioral, physiological and environmental factors that confuse attempts to isolate the controls. However, we now have cDNA probes for the genes involved in egg maturation, and we will develop specific assays to differentiate the two VGs (Obj.1). Using the probes and specific assays, the levels of VG-171 and -182 will be quantified in the blood of both dominant and subordinate queens.

A dominance hierarchy exists among the queens in polygynous colonies. If the dominant queen is removed, a second queen becomes dominant. This information provides an opportunity to determine what factor(s) prevent a subordinate queen from becoming dominant and reproductively competent. By removing the dominant queen and re-introducing the head, thorax or abdomen we can determine if some factor is present in a particular body region. This type of research has been done to determine the source of factors that are released by a queen to prevent dealation of other female ants (Vargo, 1999) f so, we will have a bioassay that will allow us to isolate and identify the factor. In addition ,preliminary investigations indicate that the blood of the dominant queen contains VG-182, but VG-171 was undetectable, as compared to the subordinate queens. One explanation may be that something is controling the utilization of both VG-182 and VG-171. If this factor is absent (or maybe present) both VG`s accumulate. For example, turning off the VG receptor could cause such a condition. Further, using queens that are producing high and low numbers of eggs and egg production measurement techniques, we will have a bioassay that evaluates factors that increase or decrease egg production. Further, we can inject or topically treat high or low egg-producing queens with extracts or factors isolated in collaboration with the Pheromone qroup, with reduced concerns with losing the test queens to exicution,, as we can maintain these queens in the same polygyne colony to measure the effects of the extracts on Vg production or receptor activity.

Objective 4. Isolate and identify the reproductive stimulant from the meconium **(Vinson).**

We have found that worker ants are attracted to the larval excrement (meconium) [larvae IFAs only release "waste" materials pupation] and they ingest the liquid contents which is fed to the queen. This stimulates the production of eggs by the queen at a time that new workers will be available to take care of them. Using the bioassay described in Objective 3, we will isolate the egg-stimulating factor. Working with the pheromone group, we will conduct bioassays and they will isolate and identify both the attractant and the reproductive stimulant. The attractant may lead to better bait attractants. Identification of the reproductive stimulant may provide us with new compounds to control reproduction or reveal new genes to exploit in the control of reproduction.

Objective 5. Quantify vitellin (Vg vitellogenin deposited in the egg) content in the different kinds of eggs **(Vinson).**

Three types of eggs are laid by fire ant queens: (1) fertilized eggs which hatch to female offspring (both workers and reproductives), (2) unfertilized eggs which hatch and become males and (3) trophic eggs which do not develop and are fed to larvae, particularly during colony founding. Determining the presence or absence of one or the other Vg and their quantity in these three kinds of eggs may provide some insight into reproductive regulation and caste determination. We hypothesize that the egg vitellin content may vary among these three types of eggs and that such differences may influence an egg's fate. If such differences occur, it suggests differential regulations of the VG genes and/or the synthesis of VGs 171 and 182.

Expected outcome/Products/Management Tools or Approaches

These projects directly address the key factors of reproduction. The results will increase our knowledge about the factors involved in stimulating or suppressing fire ant reproduction, which in turn will be useful in designing a control/management strategy for the fire ants. For example, if the Vg receptor regulates reproduction, we will have a

target for the development of hormonal mimetics, comparable to birth control pills used to control mammalian reproduction. Another possibility is that once we know the controls of reproduction, we will be able to provide the genetics program with genetic information useful in transforming microorganisms that can be used against the IFA.

Linkages with other groups
Collaboration with the Pheromone research effort is ongoing and will increase.

Time line for work to be done and anticipated technology transfer/implementation)

	1st 6 Months	2nd 6 Months
Objective 1	Initiate	Complete
Objective 2	Initiate	Cont.
Objective 3	Initiate	Cont.
Objective 4	Initiate	Cont.
Objective 5	----------	Initiate

Relevance to the Texas Imported Fire Ant Research and Management Plan (available to view on http://fireant.tamu.edu underneath the project logo in the right hand column of the home page):
This fits under the first objective "Develop an aggressive and coordinated research program designed to improve and better utilize existing fire ant management products and to develop new methods to manage fire ant populations" as part of the "pest management solution".

One year Budget and Justification for each PI

Vinson, S. B.

A. Personnel		
(1)	Principal Investigator	0
(1/2)	Research Associate	23,000
(1)	Technician	31,500
(1)	Graduate Student	16,000
(-)	Undergraduate Students	0
(1)	Ant Maintenance Tec.	9000
B. Capital Equipment		0
C. Travel		
Domestic		0
Foreign		0
D. Other Direct Costs		
Materials and Supplies		500
Publications Costs		0
Chemical Analyses		0
Services		0

Budget Justification

One Technician (Kuriachain). This person will be responsible for developing and conducting the bioassays to determine the effects of the meconium as a queen reproductive stimulant and conduct the many bioassays needed to determine the target of the stimulant (objective 4). The research here is directed to the expression of the VG genes, the production and release of the Vitellogenins, and the activation of VG receptors and uptake of the VG`s into the developing egg. This research is different from the identification of the stimulant material being conducted by the Pheromone group, but these two groups will be working together to identify the chemical nature of the stimulant. This person will also determine the types of VG in eggs of different types of queens (objective 5).

Students. Two students will be working with Dr. Keeley, Dr. Pietrantonio and Dr. Vinson to address objective 3. One student will use the DNA probes to quantitify the VG`s as a bioassay to determine the source and aid in identifying the dominate queens reproductive suppressive factor. The other student will be focused more on the difference in the levels of VG-171 and VG-182 in subordinate queens that may be related to regulation of the receptor.

One fourth 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.

Pietrantonio, P.

A. Personnel		
(1)	Principal Investigator	0
(-)	Research Associates	
(1)	Post-Doctoral Fellows	$28,000
(1/2)	Graduate Students MS	$13,000
(-)	Undergraduate Students	
(-)	Secretarial-Clerical	
B. Capital Equipment		
C. Travel		
	Domestic	$2,500
	Foreign	-
D. Other Direct Costs		
	Materials and Supplies	$9,500
	Publications Costs	$1000
	Chemical Analyses Services	$1,000

Budget Justification