ANNUAL PROGRESS REPORT
TEXAS IMPORTED FIRE ANT RESEARCH AND MANAGEMENT PROJECT

Title of the Project:

Development of novel control methods using the vitellogenin and short neuropeptide F receptors that regulate reproduction in the Red Imported Fire Ant.

Principal Investigators: Patricia V. Pietrantonio and Brad Vinson

Relevance to the Texas Imported Fire Ant Research and Management Plan

Identifying hormones, peptides or other biochemical factors that could be delivered to control the fire ant field populations or identifying the precise timing when known control methods could cause more impact leads this proposal under the category of elucidation of the biology of fire ant leading to new management methodologies.

Major accomplishments to date on:

Objective 1. Determine the Vitellogenin Receptor (VgR) localization in mated and virgin alate queens to understand Vg Receptor temporal/ spatial regulation (Pietrantonio's lab)

1. Production of the recombinant Vg Receptor antigen

In order to understand the temporal and spatial expression of the vitellogenin receptor (VgR), we propose to develop polyclonal antibodies against the Vg receptor, made in rabbits. This task is not simple because the VgR contains many regions of similarity with other Low Density Lipoprotein Receptors (LDLR), which we know are present in insects, so specificity of the antibody must be achieved to unequivocally localize only the VgR and not other related receptors of the LDLR superfamily.

We expressed a selected fragment of the receptor in bacteria as a fusion protein, which was followed by purification of the fusion protein. For this, we cloned the receptor fragment in the expression plasmid, pET32a vector (Novagen), and this recombinant plasmid was designated pET32a-*Si*VgR, which contains a partial VgR sequence with an N-terminal extension of His-tag sequences to facilitate purification of this fusion protein by affinity chromatography. A 720 bp VgR fragment (corresponding to amino acid 648 to 887 in the receptor protein) was amplified by PCR and cloned into the *Bam*HI and *Sal*I sites of pET32a vector (Novagen). Six clones were identified by restriction analysis (Fig. 1) and were sequenced to confirm their identity.

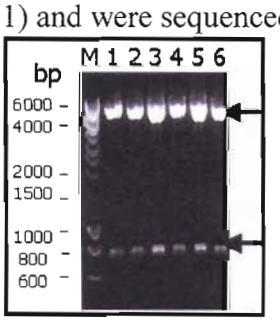

Fig 1. Expression plasmid, pET32a-*Si*VgR, was restricted with enzymes *BamHI* and *SalI*. The arrow at about 800 bp indicates the size of the expected inserted band.

After this, we tested various conditions for protein expression from this plasmid, experimenting with different temperatures and various concentrations of the promoter inducer, isopropyl-beta-*D*-thiogalactopyranoside (IPTG) in the *E. coli* strain BL21(DE3) (Fig. 2).

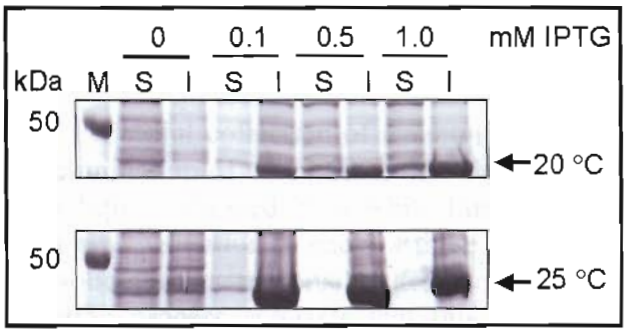

Fig. 2. Protein gel electrophoresis (10% SDS-PAGE) analysis of 10 *μl* of sample (1/200 of protein sample extracted from 25 ml of bacterial culture) from the "small scale recombinant protein induction test." The results indicated that incubation at 20°C (top panel) produced more soluble (S) recombinant protein than incubation at 25 °C (lower panel), and 1mM IPTG induced more soluble protein than 0.1 and 0.5mM IPTG. I= Insoluble protein

The results showed that the desired soluble fusion protein of about 44-kDa was induced by incubation with 1mM IPTG at 20□ for 4 h.

The fusion proteins were purified by TALON metal affinity chromatography (Clontech) in denaturing conditions according to the manufacturer's protocol (Fig. 3a) and the eluted fractions were collected and further dialyzed and concentrated in saline buffer (PBS) (Fig. 3b). Currently we are optimizing the conditions for the necessary cleavage step of the fusion protein by the enzyme enterokinase (Novagen) with different enzyme/ protein concentration ratios and temperature conditions to remove the tags present in the fusion protein. This purified and cleaved protein will be injected into rabbits as antigen to raise the polyclonal antibodies against *Si*VgR.

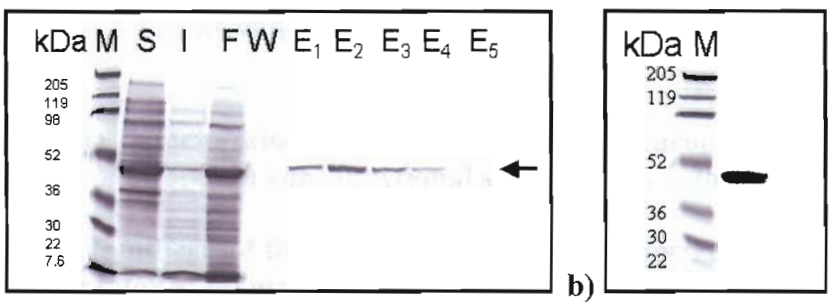

Fig 3. SDS-Page electrophoresis. a). Bacteria were treated with 6 M Guanidinium chloride (6M guanidine-HCl) and the soluble protein (S) and insoluble protein (I) were separated by centrifugation at 10000 rpm for 20 min. The fusion proteins were purified with TALON metal affinity resins and eluted with 150 mM Imidazole ($E_{1\text{-}6}$, 0.5 ml/

tube). b). The purified fusion proteins were further dialyzed and concentrated in PBS. M: Marker Molecular Weight. F: after incubated 20 min with resins. W: Buffer used to wash the resins showing that the protein is indeed attached to the resin as expected. The protein as eluted (E) from the binding resin is shown in E1-E4, four different elution fractions.

Objective 2. Determine if meconium stimulates Vg receptor transcript abundance or Vg receptor protein abundance (Vinson Lab).

Careful collection of excretory products as larvae imitate pupation revealed that the elimination of meconium, that signals that the larvae are pupating, is preceded by a clear liquid followed by a white liquid, a small tissue plug and then the meconium is released. Evaluation of each of these materials reveled that it is both the clear liquid and the solid part is responsible for the stimulation (Table 1). Careful evaluation of the pupation process suggests that this liquid arises from the malpighian tubules. Since pupation has been signaled, any JH present in the body needs to be purged from the system or metabolized. One possibility for the stimulation in egg production may be the presence of JH in the liquid.

Source	df	F Ratio	Prob > F
Model			
Time	5	5.25	< 0.001
Treatment	2	12.75	< 0.001
Time*Treament	10	7.56	< 0.001
Error	27		

Table1. Two way ANOVA on the main effects of time and treatment (depriving solid and liquid, providing both and depriving solid excreta) on queen egg production

To determine the JH titers we contacted Dr Borst (Florida) who is a renowned scientist that has developed a very sensitive method to determine JH. We collected a large sample from larvae, but he has not completed his analysis at this time. In the meantime we have collected more material and are awaiting the JH analysis to determine if the JH is responsible. In addition, we have collected more material in case we need another JH analysis. In case Dr Borst can not find any indication of JH and does not require another sample, we will determine if salts, amino acids, nitrogen waste products, or proteins are present, and if so we will determine if they have activity and then identify any active fractions.

Objective 3: Determine the ligand of the short Neuropeptide F receptor (sNPF) by *in vitro* functional studies (Pietrantonio's lab)

1. Synthesis of the HA-tagged expression construct and expression of the sNPF receptor in mammalian cells.

In order to identify a sNPF peptide that activates the putative fire ant sNPF receptor, the whole open reading frame of fire ant sNPF receptor was amplified by PCR from the plasmid p*si*NPFR 16. Four primers were used with the purpose of adding a haemaglutinin tag (HA-tag) at the N-terminus of the receptor. This PCR product was

cloned into the pcDNA3.1(-) vector (Invitrogen) and generated an expression plasmid pcDNA3.1(-)-*SiSNPFR*. The positive clones were identified by restriction analysis (Fig. 4) and sequenced to confirm their identity. This plasmid was transfected and expressed in the CHO-K1 cells as described previously (Pietrantonio *et al.*, 2005). For transfection, CHO-K1 cells were seeded into T-25 tissue culture flasks and allowed to grow overnight until 50% confluent. Cells were transfected in serum-free Opti-MEM medium (Life Technologies, Gaithersburg, MD, USA) with the cationic lipid reagent Lipofectin® (Life Technologies) (6 µl) and 2 µg of the expression plasmid according to the manufacturer's protocol. After 6 h, the lipofectin-containing medium was removed and replaced with F12K (Invitrogen) medium plus 10% fetal bovine serum without antibiotic. After 48 h growth, the cells were split into same medium but with antibiotic, 800 µg/ml GENETICIN® (Life Technologies), and continuous selection for 5 weeks and screening of clonal lines in 96 well plates.

We have selected 18 clonal cell lines. These 18 cell lines are waiting western blot screening with the anti-HA tag antibody to determine if they are expressing the receptor protein correctly, and functional receptor testing by insect sNPF peptides using a bioluminescence assay for intracellular Ca^{2+} measurement. This will determine if the expressed protein is indeed functional in the selected lines.

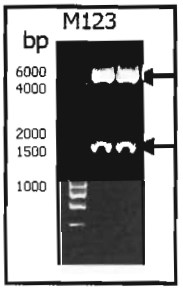

Fig. 4 . Expression plasmid, pcDNA3.1(-)-*SiSNPFR*, was restricted with *Xho*I and *EcoR*I to verify correct insertion of the receptor construct.

Goals Achieved:

Objective 1. We demonstrated that the Vg Receptor fragment can be expressed correctly in soluble form in bacteria using the pET expression vector. Thus, we have a means to produce recombinant Vg receptor protein for antibody production.

Objective 2: We have collected the larval excretion and developed a bioassay to test the effect of the excretion on the fire ant egg production, and are currently in the process of analyzing the larval excretion samples.

Objective 3: the sNPF receptor has been cloned and transfected into 18 clonal lines that await functional selection.

Development of human resources: A new Ph.D. student, Hsiao Ling Lu was recruited to conduct her PhD dissertation under this project, contributing to the development of human resources on fire ant research.

Dissemination of research:

1. International Congress/Symposia Presentations:

Invited Symposia:

Vinson, S. B., C. J. Coates, T. Haisheng, A. Rao, and T. Azizi, Sorting out some factors that influence egg production and regulate vitellogenins in the Imported Fire Ant, *Solenopsis invicta* (Hymenoptera: Formicidae). XV Congress IUSSI, July 30-Aug. 4, Washington DC. 2006.

Submitted:

Chen, M.-E. and P.V. Pietrantonio. 2006. The short neuropeptide F-like receptor from the imported fire ant (*Solenopsis invicta,* Buren). Fifth International Symposium in Insect Molecular Science. Tucson, Arizona. May 20-24, 2006. Poster presented by P.V. Pietrantonio. Abstract will be published online in the Journal of Insect Science. http://www.insectscience.org/.

Chen, M.-E. and P.V. Pietrantonio. 2006. The short neuropeptide F-like receptor from the imported fire ant (*Solenopsis invicta,* Buren). Presented as poster by P.Pietrantonio at the XV Congress of the International Union for the Study of Social Insects (IUSSI). Washington DC, July 31- August 5 2006.

Rao, A., D. Borst and S. B. Vinson. 2006. Liquid excretion of fourth instar larvae and its potential role in the fire ant reproduction. Presented as a poster by Asha Rao at the *XV International Congress of the International Union for the Study of Social Insects, Washington D.C.*

2. National Presentation.

Rao, Asha and S. B. Vinson. 2006. Larval regulation of reproduction in the red imported fire ant. Annual Red Imported Fire Ant Conference, March 28-30, Mobile AL.

4. Local Presentation

Hsiao Ling Lu. 2006. Presentation at the Department of Entomology Graduate Student Forum- Abstract. "Developing an antibody against the vitellogenin receptor from the red imported fire ant, *Solenopsis invicta,* Buren." August 22, 2006.

5. Papers published this year which were in review at the time of submission of this proposal:

Chen, Mei-Er, S.P. Holmes, and P.V. Pietrantonio. 2006. Glucose transporter 8 (GLUT8) from the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Archiv. Insect Biochem Physiol. 62*: 55-72.

Chen, Mei-Er and P.V. Pietrantonio. 2006. The short neuropeptide F-like receptor from the red imported fire ant, *Solenopsis invicta* Buren. *Archiv. Insect Biochem. Physiol. 61*:195-208.

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