Final Report:
Texas Imported Fire Ant Research Ant Management Project

Title of Project: Endocrine Regulations of Queen Reproduction in the Imported Fire Ant

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Major accomplishments to date (Sept. 1,1997 through August 31, 1999)

INTRODUCTION:

Rationale:

Endocrine regulations of reproduction in queens of the imported fire ant (*Solenopsis invicta*) are potential physiological targets for disruption in the practical control of fire ants. Identification and obstruction of the endocrine-dependent processes by antagonistic chemicals or inhibition of the synthesis, secretion or degradation of the regulatory hormones would disturb these events and interfere with egg formation. The reproductive processes and their regulatory hormones may be sufficiently specific that their inhibition would provide an insect-specific control strategy.

A related physiological event susceptible to disruption is flight muscle histolysis. Flight muscle histolysis occurs immediately upon landing following a mating flight. The wings are broken off, and digging behavior occurs for colony founding. Flight muscle histolysis appears to be endocrine dependent. It occurs either in response to increased titers of the juvenile hormone (JH) after the mating flight (Vargo and Laurel, 1994) or in response to a peptide hormone released by mating (Davis *et al*., 1989). The regulations and mechanisms related to dealation and flight muscle histolysis are ant-specific processes susceptible to disruption for fire ant control. Treatment with mimics of the hormones that promote these actions prior to mating could result in premature dealation and flight muscle degeneration that would prevent mating and new colony founding. This could be a potent new approach to fire ant control.

To understand flight muscle degeneration, it is necessary to understand the endocrine regulations of reproductive maturation. Dealation, flight muscle histolysis and vitellogenesis are all related processes and are likely all regulated by the same hormone or series of hormonal events. Vitellogenesis is better understood in insects than flight muscle degeneration, hence it is logical to elucidate the controls of vitellogenesis and use these as clues to related events for dealation. Therefore, the first research cycle has focused on vitellogenesis and developing the probes and analytical tools and techniques to evaluate vitellogenesis and its regulation in *S. invicta*.

Endocrine Regulations:

In the majority of insect species, juvenile hormone (JH) regulates reproduction by stimulating the expression of the fat body vitellogenin (VG) gene(s). The fat body then synthesizes and releases VG into the hemolymph for transport and uptake by the ovaries. JH also activates the oocytes to take up the VG where it is deposited as the yolk protein, vitellin (VN). *Drosophila* (Schonbaum *et al*., 1995) and *Aedes* (Sappington *et al*., 1996) bind and incorporate VG into the oocyte during vitellogenesis using VG receptors on the plasma membranes. These VG receptors belong to the low-density lipoprotein (LDL) receptor superfamily of vertebrates and insects.

Reproduction in the fire ant is reported to be JH dependent. VG is reportedly high in the hemolymph of alate females before the onset of vitellogenesis and decreases during vitellogenesis as the VG is taken up by the oocytes (Vargo and Laurel, 1994). Topical treatment with the JH analog,

methoprene, causes dealation and vitellogenesis despite the presence of the queen (Vargo and Laurel, 1994). This suggests that the queen pheromone suppresses reproductive maturation within the colony by suppressing JH synthesis in alate females. However, the apparent production of VG without concomitant oocyte maturation presents a physiological puzzle. Why does the virgin female produce exert the energy needed to synthesize VG when there is no oocyte maturation? Does this prepare her for a rapid onset of egg formation as soon as she is mated without delay to mobilize the VG genes and the protein biosynthetic machinery of the fat body?

Our principle hypothesis in these experiments is to determine the rate-limiting step of vitellogenesis. Three levels of regulation are proposed: (1) VG gene expression in the fat body; (2) VG biosynthesis by the fat body and (3) VG-receptor formation and the subsequent uptake of VG by the oocytes. It is likely that JH regulates one or more of these three processes; however, it is also likely that other hormones may act either alone or in concert with JH on one or more of these processes. Hymenoptera and Diptera are the two most highly evolved orders of insects, and in Diptera ecdysteroids act in coordination with JH to promote vitellogenesis (Agui *et al*., 1991; Dhadialla and Raikhel, 1994; Hagedorn, 1980; Hagedorn, 1983). Ecdysteriods could play a role in fire ants that has not been examined or considered previously. Certainly neurohormones regulate JH production and other neurohormones could have more direct effects on VG synthesis or uptake. In honeybees, presence of JH suppresses vitellogenesis but promotes foraging behavior (Robinson *et al*., 1992). JH is low in queens and egg-laying workers but highest in non-reproductive, older, foraging workers. For these reasons, it is premature to assume that fire ant reproduction is regulated solely by JH. Although JH may play the roles suggested, there may be other hormones involved that could provide alternative opportunities for disruption as a novel strategy for fire ant control.

METHODS AND MATERIALS:

Experimental Animals:

Ant colonies of *S. invicta* were maintained in the laboratory at 70/ F on a 12L:12D photoperiod. The colonies were fed honey water, cockroaches (*Blaberus discoidalis*), and diet daily. Water was given ad lib. For the age studies, reproductive pupae from a polygyne colony containing both queens and workers were removed from the brood nest and maintained separately within the colony until eclosion. Following eclosion, the female alates were sprayed with Krylon enamel paint and monitored within the colony. Newly inseminated queens were collected directly after a mating flight (College Station, TX) and held separately until ready to use.

Sample Preparation:

Hemolymph samples from *S. invicta* were collected for both the age study and the newly inseminated queen studies by a dorsal abdominal incision using 0.5 µl micro-capillary tubes (Drummond). Samples were diluted in iced-cold 1X TBS Homogenization Buffer (20 mM Tris; 0.15 M NaCl; 5 mM EDTA, pH = 5.2) containing the Complete Mini™ protease cocktail (Boehringer Mannheim) (= TBS-HB). Hemolymph protein concentrations were determined using the Micro BCA@Assay Kit (Pierce) and analyzed by SDS polyacrylamide gel electrophoresis (PAGE).

Egg samples for native analysis of *S. invicta* VN were collected from dealate females every 4 hours and sonicated in 1X TBS HB using a 50 Sonic dismembrator horn (Fisher Scientific). The egg homogenates were centrifuged at 10,000 rpm for 20 min. at 4/ C to pellet the debris and the supernatant was further purified through glass wool. The samples were stored at 4/ C and used within 24 hours for PAGE analysis.

SDS Polyacrylamide Gel Electrophoresis (PAGE):

Hemolymph samples were brought to a final protein concentration of 8 µg (unless otherwise specified) and diluted in an equal volume of denaturing sample buffer (2.3% SDS; 63 mM Tris-Cl, pH=6.8, 6.3% glycerol, 0.005% bromophenol blue, 1% NaCl; 5% β–mercaptoethanol). Proteins were analyzed by SDS-PAGE (Laemmli, 1970). The proteins were separated on a 6-16% separating gel with a 4.5% stacking gel for approximately 2 hours at 5 watts. The protein bands were stained using Gel Codea Blue Stain and destained with distilled water.

Molecular Weight Determination:

The molecular weight of the native VN was estimated using the Nondenatured Protein Molecular Weight Marker Kit (Sigma, St. Louis MO). Electrophoresis of egg homogenates was performed under non-denaturing conditions (sample buffer lacked SDS and β–mercaptoethanol; gels lacked SDS) using a series of polyacrylamide gels (5.5%, 6.0%, 7.0%, 7.5%, 8.0%, 9.0%, and 10%). A standard curve was determined using a Ferguson Plot which plots 100 [log (R_f * 100)] values against the gel concentration as percent (Sigma Tech. Bulletin No. MKR-137, 10-86). The molecular weight markers (Sigma) were bovine α-Lactalbumin (M_r = 14,200); bovine erythrocytes carbonic anhydrase (M_r = 29,000), chicken egg albumin (M_r = 45,000), bovine serum albumin (monomer M_r = 66,000, dimer M_r = 132,000), and jack bean urease (trimer M_r =272,000; hexamer M_r = 545,000). The molecular weight determination of the VG and VN subunits was accomplished by SDS-PAGE (6-16% gradient) using BioRad broad-range, molecular weight standards.

Antibody production:

Egg samples were collected from queen *S. invicta* and handled similar to the native egg samples described above. Egg samples were collected every 4 hours and sonicated in 1X TBS-HB and stored at 4/ C for up to 24 hours. The egg homogenates were pooled after 24 hours, microconcentrated (Amicon), and placed in denaturing sample buffer (2.3% SDS; 63 mM Tris-HCl, pH=6.8, 6.3% glycerol, 0.005% bromophenol blue, 5% β–mercaptoethanol). After denaturation at 65/ C, the samples were stored at -20/ C until ready for SDS-PAGE analysis. The polyacrylamide gel slices were micro-eluted using a Hoeffer Gel Eluter. The eluted protein was micro-concentrated and pooled. Injections of VN into New Zealand white rabbits was performed by Laboratory Animal Resources and Research (Texas A & M University, College Station, TX). Booster injections were given after 3 and 6 weeks following immunization. The serum was collected a week following the last immunization and stored at -80/ C.

Immunoblotting:

Egg homogenates and hemolymph samples from queen, alate and male polygyne *S. invicta* were subjected to SDS-PAGE as described above. The gel containing the separated proteins was transferred overnight at 15 mA to nitrocellulose at 4/ C. The membrane was exposed to *S. invicta* anti-VN and detection of antigen/antibody interaction was accomplished using Immunopurea Goat Anti-Rabbit IgG, (H+L) Peroxidase conjugated secondary antibody (Pierce). Supersignala West Pico Chemiluminescent Substrate (Pierce) was used to visualize the reaction.

RT-PCR of VG and VG Receptor Fragment:

Total fat body and ovary RNA was extracted from female *S. invicta* at 10 days post-insemination using the guanidine-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi,

1987; Puissant and Houdebine, 1990). Reverse transcription of total fat body and ovary RNA was accomplished using the GeneAmpa PCR Core Kit (Perkin Elmer) according to the manufacturer's recommendations.

Isolation of the 1.2 kb VG Fragment and the 2.4 kb VG Receptor Fragment:

The fat body cDNA template (≤1.0 µg) obtained as described above was used to amplify a 1.2 kb region of the VG gene using degenerate primers designed from two hymenopteran species (*Athalia rosae* and *Pimpla nipponica*) for which the complete VG sequence is known. The GeneAmpa PCR Core Kit (Perkin-Elmer) was used for amplification according to the manufacturer's recommendations. Bands were analyzed on 1% agarose gel and visualized with ethidium bromide staining. The 1.2 kb fragment was isolated, cloned into pGEMa -T Easy (Promega) and sequenced. The cloned sequence was compared with other genes available through the database of the National Center for Biotechnology Information BLAST e-mail server.

Isolation of the 2.4 kb VG Receptor Fragment:

The same methodology used for amplification of the VG gene was also incorporated for isolation of the 2.4 kb VG receptor fragment. Amplification of ovary cDNA (≤1.0 µg) was accomplished using two degenerate primers designed from the mosquito, *Aedes aegyti* and the fruit fly, *Drosophila melonogaster* VG receptor gene sequences. The GeneAmpa PCR Core Kit (Perkin Elmer) was used for amplification according to the manufacturer's recommendations. Bands were analyzed on 1% agarose gel and visualized with ethidium bromide staining. The 2.4 kb fragment was isolated, cloned into pGEMa -T Easy (Promega) and sequenced. The cloned sequence was compared with other genes available through the database of the National Center for Biotechnology Information BLAST e-mail server.

Northern Blot Analysis of the VG gene and the VG Receptor:

The size and specificity of the VG gene and VG receptor were verified by northern blot analysis. Total RNA for VG gene and VG receptor analysis was extracted, as described above, from the fat body and ovaries of 10-day-old queens; alate females and the fat body of males. The RNA was denatured with methylmercuric hydroxide (Bailey and Davidson, 1976) and separated by electrophoresis in 1.2% agarose. The RNA was transferred to a positively charged Zeta-Probe nylon blotting membrane (BioRad) using 10 mM NaOH as the transfer medium and illuminated with UV (300 nm) to fix the RNA to the membrane. The membrane was hybridized overnight at 42/ C with the appropriate ^{32}P-labeled probe using Ultra-Hyb (Ambion).

RESULTS

VG and VN - Properties:

Native VN was isolated from eggs by non-denaturing polyacrylamide electrophoresis and found to consist of a single protein of 350 kDa. A second band was occasionally observed on the gels at approx. 175 kDa, which suggested that VN sometimes degraded into two nearly identical subunits. Subsequent studies of hemolymph proteins and proteins in the incubation medium from *in vitro* fat body indicate that VG consisted of two similarly sized subunits – of 175 and 180 kDa. We do not observe any comparable bands present in hemolymph from males or in incubation medium from fat body of males *in vitro*.

VG levels were monitored at various ages and stages of development by SDS-PAGE. No VG was observed in pupae; alate, virgin females or in workers. Newly mated queens showed a weak band of VG by 24 h after mating. The VG band was strong 2 days after mating and highly abundant at 6 days. We are currently monitoring the abundance of VG through 60 days after mating, and we are comparing relative VG synthesis between monogyne and polygyne queens.

A 50-kDa polypeptide was also highly abundant in the hemolymph of dealate females that were producing eggs in a polygyne colony. The 50-kDa polypeptide was abundant in workers but absent from pupae and from males. It was sometimes present in alate females. The polypeptide appeared to be sex-specific but was not found in eggs, nor was it synthesized by the fat body *in vitro*. The significance of this abundant, female-specific protein is unknown but its high abundance and unusual presence makes it of interest to examine more carefully.

A polyclonal rabbit antibody was developed to the VN from fire ant eggs. The anti-VN reacts with both VN from egg homogenates and VG from queens and older alate females that show some ovary development. Anti-VN does not react with any proteins present in male hemolymph.

VG gene isolation:

Isolation of the VG gene was undertaken based on PCR using degenerate primers to conserved sequences from the VG genes of two wasp species, *Athalia rosae* and *Pimpla nipponica*. The primers were used in RT-PCR of fat body mRNA from egg-producing queens of *S. invicta*. A 1.2-kb sequence was obtained that shows strong amino acid homology with other known VGs. The sequence was used to determine sex and tissue specificity for expression of the gene. Northern blot analysis of RNA from fat body of males and newly mated queens and from ovary of newly mated queens showed abundant mRNA in the fat body of newly mated queens. Ovaries showed only a weak band, and no band was found for fat body of males.

This sequence is being used as a probe to isolate the full length VG gene from a *Solenopsis invicta* genomic library (courtesy of Dr. L.C. Skow, College of Vet. Med., TAMU). Once available, a cDNA sequence for the gene will be used as a probe to evaluate sensitivity of the fat body to hormonal treatments by quantitative assessment of VG-mRNA using slot-blot techniques.

Vg receptor gene:

Degenerate primers were developed based on conserved sequences from the VG-receptor sequences of *Drosophila* and *Aedes* and used in RT-PCR of mRNA from ovary of egg-producing queens of *S. invicta*. The RT-PCR produced a 2.4-kb sequence that had high homology with a large number of genes belonging the low-density lipoprotein receptor superfamily. Using the 2.4 kb sequence as a probe, total RNA from fat body and ovaries was analyzed by northern blots to determine if the VG-receptor mRNA were present. Both tissues showed positive response to the

probe. It was anticipated that the VG-receptor would be specific to the oocytes in the ovaries. However, LDL-receptors in locusts were expressed in the fat body, brain and midgut as well as the oocytes (Dantuma *et al*., 1999). It is possible that the VG-receptor of the oocytes is a general LDL receptor and not tissue specific. Alternatively, related but different LDL-receptor genes are expressed in both the fat body and oocytes and produce proteins with functions unique for each tissue. The entire gene sequence for this LDL-receptor is being determined from the *S. invicta* genomic library.

In vitro VG biosynthesis:

An *in vitro* organ culture system has been established for *S. invicta* fat body. Fat body from alate females, males and workers remained alive and synthesized protein for up to 48 hr in an incubation medium consisting of salts and an amino acid composition comparable to that of *S. invicta* hemolymph as determined by Consoli and Vinson (personal communication). Fat body protein synthesis was measured by [^{35}S]methionine incorporation into released proteins. Protein synthesis remained nearly constant during the 48-hr period. Anti-VG will allow specific recovery of newly synthesized VG for evaluation of VG translation in response to various endocrine regimes.

SIGNIFICANCE

Research during the first two years of this project was designed to provide us a foundation of knowledge and the experimental tools to address the long-range objectives of this research. Any organism has its idiosyncrasies that must be acknowledged, elucidated and accounted for in designing physiological investigations that measure real-world situations. Social insects, because of their tightly controlled caste system regulated by exposure to concentrations of a queen pheromone, are especially difficult to synchronize to ensure replicate studies starting at the same point in the reproductive cycle.

Studies of the properties of IFA VN, VG and the timing of VG synthesis in alate, virgin females and newly mated queens have provided critical baseline information concerning the reproductive cycle in females of IFA. Furthermore, our studies are predicated on using the powerful analytical tools of molecular biology. Fire ants are small insects and are, therefore, difficult to handle. It is tedious to collect sufficient amounts of IFA tissues for physiological analyses and studies. Hence, the necessity to use the ultra-sensitive technologies of molecular biology to examine the physiological events of interest.

The first two years of the project have been spent identifying the genes for VG and for the VG receptor, elucidating their structural properties and developing assay procedures for their detection. During the first two years we have: (1) isolated the VG gene, (2) isolated a gene in the low-density lipophorin-receptor superfamily that is the putative VG receptor, (3) established an in vitro assay system for the IFA fat body that will allow us to monitor VG synthesis and test its sensitivity to specific hormones and (4) developed an antibody to VG and confirmed its specificity. The anti-VG will allow us to specifically detect circulating, hemolymph VG and VG synthesized by the fat body in vitro. These isolated genes, the in vitro fat body culture system and the VG antibody comprise the probes and technical tools that will allow us to evaluate the endocrine controls of IFA reproduction at the levels of specific gene expression and the synthesis of gene products. The information and experience that we have acquired to date concerning IFA reproduction, combined with the development of the molecular tools and techniques, position us for undertaking the critical experimental studies that will demonstrate the specific controls of IFA reproduction.

This research will elucidate both the endocrine regulators of egg production in queens and

flight muscle histolysis. Identification of these hormones will suggest chemicals and strategies for their most efficacious application that can be used to disrupt these processes to inhibit both colony formation and reproduction for more effective control of the IFA. JH most likely plays the key role in IFA reproduction. But understanding if, and how, JH interacts with other hormones, such as ecdysteroids and/or neuropeptides, and determining the rate-limiting step in IFA reproduction (VG gene expression, VG synthesis by the fat body, or VG uptake by the oocytes) is crucial to effective chemical control of the IFA. New ecdysteroid antagonists and JH mimics have been developed in recent years, and effective peptidomimetics, are on the horizon. For example, mimics of allatostatins that shut down JH synthesis are being developed. Therefore, comprehensive elucidation of all the interactive factors controlling IFA reproduction is timely and essential to identify potential weakpoints susceptible to manipulation. Our approach to IFA control will identify these weakpoints and suggest either novel chemicals or more effective uses of available technology to interrupt IFA reproduction.

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