Gene Structure and Expression of the Glutathione S-transferase, *SiGSTS1*, from the Red Imported Fire Ant, *Solenopsis invicta*

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The structural organization and developmental expression of a previously described glutathione S-transferase cDNA from the red imported fire ant, *Solenopsis invicta*, were elucidated. The gene was previously named *Solenopsis invicta*, glutathione S-transferase, sigma class no. 1 (*SiGSTS1*). Comparison of genomic and cDNA sequences showed that the gene was comprised of 5 exons and 4 introns. All of the introns possessed the 5′GT and 3′AG splicing sites characteristic of eukaryotes. The comparative C_T method of quantitative-polymerase chain reaction was employed to examine the developmental expression of the *SiGSTS1* transcript in monogyne and polygyne *S. invicta*. Polygyne queen and late instars exhibited 3.5- and 4.7-fold increased expression of *SiGSTS1*, respectively, compared with pupae. Early instars (13.1-fold) and workers (9.6-fold) exhibited the highest, and statistically significant, levels of expression of all polygyne developmental stages examined. A similar pattern of expression was observed for the monogyne social form. However, the queen showed the lowest expression level, followed by pupae (1.2-fold), late larvae (5.8-fold), early larvae (9.4-fold), and workers (10.1-fold). No differences were observed in the *SiGSTS1* gene sequences between fire ant social forms. Although obviously developmentally expressed, the function of *SiGSTS1* in *S. invicta* is not known. Arch. Insect Biochem. Physiol. 61:239–245, 2006.   Published 2006 Wiley-Liss, Inc.[†]

KEYWORDS: *Solenopsis invicta*; glutathione S-transferase; gene expression

INTRODUCTION

The red imported fire ant, *Solenopsis invicta* Buren, was introduced into the United States from South America in the early 1900s and has since spread to infest more than 128 million hectares in the southeastern United States and parts of California, Arizona, and New Mexico (Williams et al., 2001). This aggressive, territorial ant adversely impacts arthropod biodiversity in infested areas (Porter and Savignano 1990; Porter et al. 1991), causes economic losses in the cattle industry (Barr et al., 1994), damages agricultural commodities (Lofgren,

1986), hampers interstate commerce of nursery stock as a result of quarantine regulations, and even represents a significant risk to human health (Williams et al., 2001). Total costs associated with fire ant control and damage have been estimated at 0.5 to 1 billion dollars annually (Thompson et al., 1995). Despite near complete reliance on insecticides for control of *S. invicta*, very little information is available concerning insecticide detoxification in this pest.

Glutathione S-transferases (GSTs) comprise an enzyme superfamily associated with a variety of functions including protection from reactive oxygen

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species, maintenance of a reductive environment for thiolated proteins, biosynthesis of prostaglandins, and glutathione conjugation of ligands (endogenous and exogenous), which facilitates excretion of these compounds by increasing their solubility (Kanaoka et al., 1997; Sheehan et al., 2001). Thus, GSTs are considered an important detoxification enzyme system (Yu, 1996) and have been shown to be involved in pesticide detoxification (Edwards et al., 2000; Abel et al., 2004), and insecticide resistance in insects (Wei et al., 2001). We recently purified the first glutathione S-transferase (GST) from adult workers of *S. invicta* by affinity chromatography and preparative isoelectric focusing (Valles et al., 2003). Degenerate oligonucleotide primers were designed from sequenced sections of the purified enzyme and subsequently used to identify and characterize the corresponding cDNA. BLAST analysis revealed significant identity with Sigma class GSTs. The enzyme was named, *Solenopsis invicta*, glutathione S-transferase, Sigma class no. 1 (*SiGSTS1*). Here, we continue the characterization of *SiGSTS1* by elucidating the gene structure and developmental expression in *S. invicta*.

MATERIALS AND METHODS

Ants

Queenright polygynous or monogynous *S. invicta* colonies were excavated from areas in Gainesville, FL. Ants were separated from the soil and transferred to rearing trays using the floating technique described previously (Jouvenaz et al., 1977). Colony social form was determined by genotyping a sample of worker ants (20) from each nest at the *Gp-9* locus (Valles and Porter, 2003). Five developmental stages were used for the expression studies, including queen, early larvae (first and second instars), late larvae (third and fourth instars), white pupae, and workers. Larval stages were determined as described by Petralia and Vinson (1979).

Determination of Gene Structure

To determine the gene sequence of *SiGSTS1*, PCR was conducted with genomic DNA using a

series of overlapping oligonucleotide primers (p27A, p34; p33, p28A; p300, p301) designed from the transcript (Table 1). Genomic DNA was used as the template for PCR and isolated from monogyne and polygyne *S. invicta* with the PUREGENE DNA isolation kit (Gentra, Minneapolis, MN). Construction of the gene was accomplished by creating 3 overlapping fragments by PCR with genomic DNA using 3 pairs of oligonucleotide primers (within exons) that were designed from the cDNA sequence. PCR was conducted in an MJ Research PT-100 thermal cycler using the following temperature regime: 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 15 sec, 53°C for 15 sec, 68°C for 0.5–2 min, and one cycle at 68°C for 5 min. Amplicons produced were subsequently ligated into pGEM-T Easy, transformed into JM109-competent cells (Promega, Madison, WI), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). To determine the structural organization of the *SiGSTS1* gene, genomic sequence (accession number DQ191801) was compared with the cDNA sequence (accession number AY255670). Comparative analysis of the genomic and cDNA sequences was accomplished with the SPIDEY application of the NTI Vector software (Invitrogen).

SiGSTS1 Expression by Quantitative PCR

SiGSTS1 expression was quantified using real-time quantitative PCR by the comparative C_T

TABLE 1. List of Oligonucleotide Primers Used in This Study

Primer name	Nucleotide position[a]	Sequence (5′ to 3′ orientation)
QSiGSTS1F	338–362	CCATGGAGATTGATGCCACTATTGA
QSiGSTS1R	401–424	TGAAAGCAGGATCTTGTTCCCAAT
QSiGSTS1ProbeFAM	371–392	ACGATATAAGACAGGCTTTATC
Q18SF	501–525	CCCGTAATCGGAATGAGTACACTTT
Q18SR	577–599	ACGCTATTGGAGCTGGAATTACC
Q18SProbeFAM	549–565	CACCAGACTTGCCCTCC
p33	85–111	ATGCCGACATATAAGCTAACGTATTTC
p34	667–690	CAGTTGTGTCTTGGGTCGTTTCTC
p85	1,064–1,088	GCTAGCTGGCATCGTTTATGGTTAG
p27A	1–24	AGTCGTTCACGATTCACGAACAAC
p28A	876–900	GGATATTGTGCTAAAGCAACATCGC
p300	931–960	TGAATGAAAAAGATTTTATTTCCTTTCTAT
p301	827–858	CTGCAACCTAACAGTTAATGTTATTACATAAC

[a]Positions correspond to the transcript.

method (Livak and Schmittgen, 2001). This method utilizes an endogenous reference and treatment calibrator to determine the relative quantity of a target transcript. One crucial requirement for use of this method is a validation experiment to demonstrate that reverse transcription efficiencies of the target and reference genes are approximately equal. We chose the 18S ribosomal RNA gene (Valles and Pereira, 2003; GenBank accession number: AY334 566) as our endogenous reference. Nucleotide sequences for the *SiGSTS1* and 18S ribosomal genes from *S. invicta* were submitted to the Assays-by-Design Service at Applied Biosystems Inc. (Foster City, CA) for synthesis of TaqMan MGB probes and primers for each respective gene. Nucleotide sequences and gene positions for each of the TaqMan probes and oligonucleotide primers are provided in Table 1. Both probes utilized the reporter dye, FAM (6-carboxyfluorescein), attached to the 5′ end and a dark quencher, DABCYL (4-(4′-dimethylamino-phenylazo)benzoic acid) attached to the 3′ end. The probe for the target gene, *SiGSTS1*, was designed to span intron 3 as an additional measure to avoid amplification of genomic DNA (Fig. 1).

First, an experiment was conducted to deter-mine the suitability of the *18S* ribosomal gene as a reference for the *SiGSTS1* gene. RNA (0.01, 0.1, 1, 10, and 100 ng) extracted from workers was incubated with 1 U of amplification grade DNAse I (Invitrogen, Carlsbad, CA) for 15 min at room temperature. The DNAse activity was terminated by addition of EDTA to a final concentration of 2.27 mM and heating to 65°C for 10 min. cDNA synthesis was subsequently completed with Super-Script II reverse transcriptase (Invitrogen) and oligonucleotide primers, p34 and p85, for *SiGSTS1* and *18S*, respectively (Table 1). These primers were downstream of the area amplified during the subsequent quantitative PCR. Reverse transcription was completed in a thermalcycler at 45°C for 30 min and terminated by heating to 70°C for 15 min. Quantitative PCR was subsequently completed in an Applied Biosystems, Inc., Prism Sequence Detection System 7000 in a 25-µl reaction volume with the corresponding probe/primer combination (Table 1) using the TaqMan Universal PCR Master Mix (Applied Biosystems) following the temperature regime: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min. Successful DNAse I treatments were confirmed by lack of

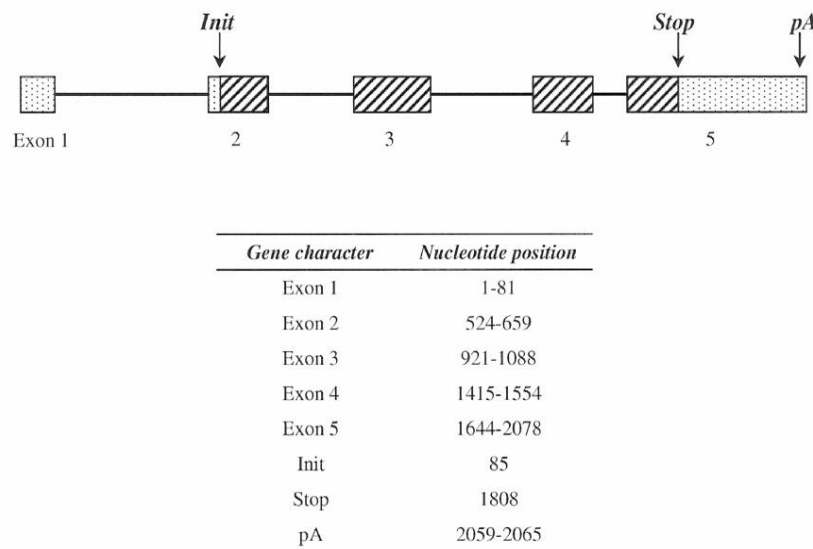| Gene character	Nucleotide position
Exon 1	1-81
Exon 2	524-659
Exon 3	921-1088
Exon 4	1415-1554
Exon 5	1644-2078
Init	85
Stop	1808
pA	2059-2065

Fig. 1.   Schematic representation of the *SiGSTS1* gene and tabulated locations of relevant characters. Genomic architecture of the regions encoding *SiGSTS1*. Init, Stop, and pA denote the approximate locations of the initiation codon, stop codon, and polyadenylation signal, respectively. Exons are indicated by boxes. Cross-hatched areas represent *SiGSTS1* coding regions; stippled areas represent the 5′ and 3′ untranslated regions.

amplification in quantitative PCR among identical samples without reverse transcription.

Once the suitability of the *18S* gene as a reference was established (Fig. 2), the relative quantity of the *SiGSTS1* transcript was determined in polygyne and monogyne queens, early and late larvae, workers and pupae by this same method. Total RNA was extracted using the Agilent Technologies (Wilmington, DE) RNA isolation protocol. Ten to 20 mg of ants or brood, or a single queen, was used as the RNA source. The RNA was quantified spectrophotometrically and diluted to 10 ng/μl for analysis.

Three replications of each experiment were conducted. Change in critical threshold (ΔC_T) was determined by subtracting the *SiGSTS1* C_T from the *18S* C_T value. $\Delta\Delta C_T$ was then determined by subtracting ΔC_T from the ΔC_T of the calibrator (treatment [developmental stage] with the lowest expression level [highest ΔC_T]). The calibrator was the treatment with the lowest expression level (i.e., queen stage for monogyne fire ants and pupal stage for the polygyne fire ants). The relative expression ($2^{-\Delta\Delta C_T}$) of *SiGSTS1* was calculated and non-overlapping confidence intervals were used

to indicate significant differences between treatments (Livak and Schmittgen, 2001).

SiGSTS1 Cloning and Sequencing From Polygyne and Monogyne *S. invicta*

S. invicta exists in two distinct social forms, monogyne and polygyne. Valles et al. (2003) had previously cloned and sequenced the *SiGSTS1* gene exclusively from polygyne fire ant colonies. To determine if there were any differences between monogyne and polygyne *SiGSTS1*, individual worker ants (about 45) from verified monogyne and polygyne colonies (Valles and Porter, 2003) were used to amplify the *SiGSTS1* gene by One-Step RT-PCR with oligonucleotide primers p27A and p28A (Table 1). The 900-bp amplicon was cloned into the pCR4 vector, transformed into TOP10 competent cells (Invitrogen), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). Three clones derived from each social form were sequenced in entirety.

Glutathione *S*-Transferase Activity

Glutathione S-transferase (GST) activity was measured in the different developmental stages (queen, early larvae [first and second instars], late larvae [third and fourth instars], white pupae, and workers) from a monogyne colony with 1-chloro-2,4-dinitrobenzene (CDNB) as described previously (Habig et al., 1974). The different fire ant developmental stages were homogenized in 0.1 M sodium phosphate buffer, pH 6.5, filtered through 2 layers of cheesecloth, and centrifuged at $105,000g_{Max}$ for 1 h. The supernatant was used as the enzyme source. The 3-ml reaction mixture contained 1 ml of 15 mM glutathione and 0.025–0.05 mg of soluble fraction protein in 2 ml of homogenization buffer. The mixture was incubated at 25°C for 3 min, then 0.02 ml of 150 mM CDNB in ethylene glycol monomethyl ether was added and mixed. The change in absorbance at 340 nm was measured on a Varian Model 3 Bio uv/vis spectrophotometer equipped with a diffuse reflectance accessory against a reference containing buffer, CDNB, and glutathione. En-
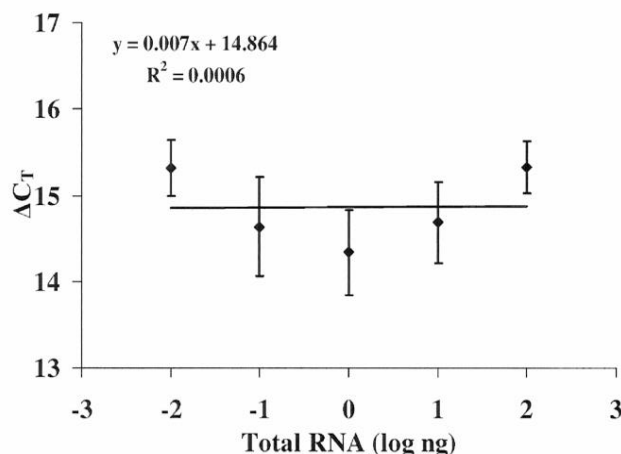

Fig. 2.　Plot of the ΔC_T for *SiGSTS1* (target gene) and *18S* (reference gene) over a range of RNA concentrations. A slope of <0.1 indicates that reverse transcription efficiency for these two genes, over the range indicated, is approximately equal, thereby validating use of the *18S* gene as reference for the *SiGSTS1* target gene (Livak and Schmittgen, 2001).

zyme activity was calculated with the molar extinction coefficient of 9.6 mM^{-1}cm^{-1} for the product, S-(2,4-dinitrophenyl)glutathione.

RESULTS AND DISCUSSION

Comparative analysis of the genomic and cDNA sequences of *SiGSTS1* revealed 5 exons spanning 2 kb (Fig. 1). All of the introns possessed the 5'GT and 3'AG splicing sites characteristic of eukaryotes (Breathnach and Chambon, 1981). Exon 1 was comprised entirely of an untranslated region, while exons 2, 3, 4, and 5 comprised the majority of the translated nucleotide sequence of *SiGSTS1*. Exon 2 contained the translation initiation site and exon 5 contained the stop site and a polyadenylation signal.

Efficiency of reverse transcription for the *S. invicta SiGSTS1* and *18S* genes was determined to be similar within the range of RNA quantities used in our experiments (Fig. 2); the absolute value of the slope (0.007) of log RNA versus ΔC_T was less than 0.1 as required for comparative C_T method validity. Reference and target genes must exhibit relatively similar reverse transcription efficiencies for the comparative C_T method to be valid (Livak and Schmittgen, 2001). Thus, utilization of the comparative C_T method to determine relative gene expression of the target gene, *SiGSTS1*, with the *18S* gene provided a suitable reference based on the defined requirements.

In polygyne *S. invicta*, *SiGSTS1* expression was lowest in the pupae, and so this developmental stage was used to calibrate expression in other stages (Livak and Schmittgen, 2001). Polygyne queen and late larvae exhibited 3.5- and 4.7-fold higher expression of *SiGSTS1*, respectively, compared with pupae, although no significant difference was noted between queens and late larvae (Fig. 3). Expression in early larvae (13.1-fold) and workers (9.6-fold) exhibited the highest, and statistically significant, levels of expression of all polygyne developmental stages. A similar pattern of expression was observed for the monogyne social form. However, the queen showed the lowest expression level, followed by pupae (1.2-fold), late larvae (5.8-fold), workers (10.1-fold), and early larvae (9.4-fold).

Interestingly, a general decreasing trend in *SiGSTS1* transcript corresponded to developmental
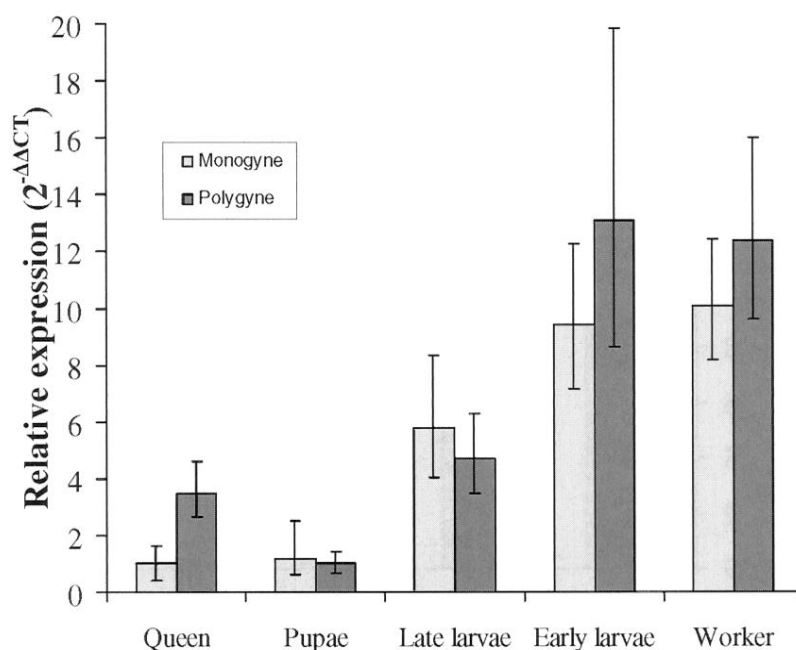

Fig. 3.   Relative expression ($2^{-\Delta\Delta C_T}$) of *SiGSTS1* in different developmental stages of monogyne and polygyne fire ant colonies. Error bars represent confidence intervals as calculated by the comparative C_T method (Livak and Schmittgen, 2001). Means with overlapping confidence intervals are not significantly different.

progression. The *SiGSTS1* transcript level decreased from early larvae through late larvae to pupae (Fig. 4), while a corresponding increase in general GST enzyme activity (CDNB conjugation) occurred for the same developmental stages. This result supports our earlier conclusion that a number of GST isozymes other than *SiGSTS1* are likely present in *S. invicta* because despite a decrease in transcript level, general GST activity increases. The relationship between transcript level, GST activity, and division of labor was somewhat unexpected. Because GSTs are known to play crucial roles in xenobiotic detoxification, as well as provide important antioxidant protective effects, we expected the late larvae and workers to possess the highest activities and transcript levels. Fourth instar larvae (included in our late larvae category) do all of the digestion of solid food for the entire colony (Petralia and Vinson, 1978) and workers must leave the relative protection of the nest to collect food (solid and liquid) for the colony. Thus, these two stages would, ostensibly, need more protection from xenobiotics and oxidative stress due to higher environmental exposure. Workers did possess the highest *SiGSTS1* transcript level and general GST activity. However, late larval GST activity and *SiGSTS1* transcript levels were intermediate. Papadopoulos et al. (2004) reported that GST activity (CDNB-conjugation) was highest

in adult *Apis mellifera macedonica* compared with other developmental stages examined. They also found dramatic shifts in the expression of GST genes among developmental stages. These data further support our hypothesis that *S. invicta* possesses different GST genes that are differentially expressed during development.

Finally, because a genetic difference between polygyne and monogyne fire ants has been identified at the *Gp-9* locus (Krieger and Ross, 2002), we wanted to determine if there were any corresponding differences in the *SiGSTS1* gene between social forms. Valles et al. (2003) had reported the cDNA sequence for *SiGSTS1* from the polygyne social form only. No differences were observed in the *SiGSTS1* gene sequences between fire ant social forms. Indeed, the monogyne sequence was identical to the polygyne sequence we reported earlier (Valles et al., 2003; accession number AY255670).
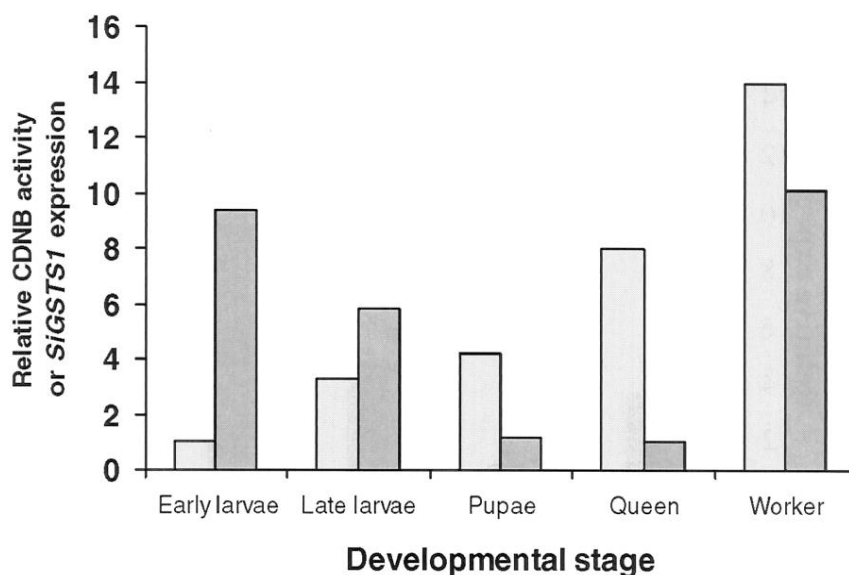
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Fig. 4.   Relative CDNB GST activity (light-colored bars) and relative *SiGSTS1* expression (darker-colored bars) in different developmental stages of *S. invicta*.

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LITERATURE CITED

Abel EL, Brammler TK, Eaton DL. 2004. Biotransformation of methyl parathion by glutathione S-transferases. Toxicol Sci 79:224–232.

Barr CL, Drees BM, Cohen ND, Reagor JC, Eugster AK, Sprott LR, Bade DH, Cornforth GC, Vinson SB. 1994. Final report of the Texas veterinarian survey: impact of red imported fire ants on animal health. Tex Agric Ext Serv Bulletin: Feb. 1994.

Breathnach R, Chambon P. 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu Rev Biochem 50:349–383.

Edwards R, Dixon, DP, Walbot V. 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci 5:193–198.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Jouvenaz DP, Allen GE, Banks WA, Wojcik DP. 1977. A survey for pathogens of fire ants, *Solenopsis* spp., in the southeastern United States. Fl Entomol 60:275–279.

Kanaoka Y, Ago H, Inagaki E, Nanayama T, Miyano M, Kikuno R, Fujii Y, Eguchi N, Toh H, Urade Y, Hayaishi O. 1997. Cloning and crystal structure of hematopoietic prostaglandin D synthase. Cell 90:1085–1095.

Krieger MJB, Ross KG. 2002. Identification of a major gene regulating complex social behavior. Science 295:328–332.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Lofgren CS. 1986. The economic importance and control of imported fire ants in the United States. In: Vinson SB, editor. Economic impact and control of social insects. New York: Praeger. p 227–256.

Papadopoulos AI, Polemitou I, Laifi P, Yiangou A, Tananaki C. 2004. Glutatione S-transferase in the developmental stages of the insect *Apis mellifera macedonica*. Comp Biochem Physiol 139C:87–92.

Petralia RS, Vinson SB. 1978. Feeding and larvae of the imported fire ant, *Solenopsis invicta*: behavior and morphological adaptations. Ann Entomol Soc Am 71:643–648.

Petralia RS, Vinson SB. 1979. Developmental morphology of larvae and eggs of the imported fire ant, *Solenopsis invicta*. Ann Entomol Soc Am 72:472–484.

Porter SD, Savignano DA. 1990. Invasion of polygyne fire ants decimates native ants and disrupts arthropod community. Ecology 71:2095–2106.

Porter SD, Bhatkar AP, Mulder R, Vinson SB, Clair DJ. 1991. Distribution and density of polygyne fire ants (Hymenoptera: Formicidae) in Texas. J Econ Entomol 84:866–874.

Sheehan D, Meade G, Foley VM, Dowd CA. 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J 360:1–16.

Thompson SC, Jones DB, Semevski FN, Semenov SM. 1995. Fire ant economic impact: extending Arkansas' survey results over the south. In: Vinson SB, Drees BM, editors. Proceedings of the Imported Fire Ant Research Conference, San Antonio, TX. p155–156.

Valles SM, Pereira RM. 2003. Use of ribosomal DNA sequence data to characterize and detect a neogregarine pathogen of *Solenopsis invicta* (Hymenoptera: Formicidae). J Invertebr Pathol 84:114–118.

Valles SM, Porter SD. 2003. Identification of polygyne and monogyne fire ant colonies (*Solenopsis invicta*) by multiplex PCR of *Gp-9* alleles. Insect Soc 50:199–200.

Valles SM, Perera OP, Strong CA. 2003. Purification, biochemical characterization, and cDNA cloning of a glutathione S-transferase from the red imported fire ant, *Solenopsis invicta*. Insect Biochem Mol Biol 33:981–988.

Wei SH, Clark AG, Syvanen M. 2001. Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly *Musca domestica*. Insect Biochem. Mol Biol 31:1145–1153.

Williams DF, Collins HL, Oi DH. 2001. The red imported fire ant (Hymenoptera: Formicidae): an historical perspective of treatment programs and the development of chemical baits for control. Am Entomol 47:146–159.

Yu SJ. 1996. Insect glutathione S-transferases. Zoological Studies 35: 9–19.