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Manipulation of the Solenopsis Invicta Virus-1 by RNA Interference in the Red Imported Fire Ant Solenopsis Invicta (Hymenoptera: Formicidae), and Examination of Differences in Resulting Micro RNA Expression

Patrick Rydzak

MANIPULATION OF THE *SOLENOPSIS INVICTA* VIRUS-1 BY RNA INTERFERENCE IN THE RED IMPORTED FIRE ANT *SOLENOPSIS INVICTA* (HYMENOPTERA: FORMICIDAE), AND EXAMINATION OF DIFFERENCES IN RESULTING MICRO RNA EXPRESSION

by

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-Patrick Rydzak

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The University of Texas at Tyler
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The red imported fire ant, *Solenopsis invicta* (Hymenoptera, Formicidae), is an invasive ant species from South America to the United States and is responsible for approximately six billion dollars (U.S.) every year. In 2004, as part of an effort to find a self-sustained, long term biological control agent against *S. invicta*, researchers discovered the first ever known virus to infect *Solenopsis sp.*, the *Solenopsis invicta* virus-1 (SINV-1). RNA interference (RNAi) is a known regulator of biological systems in insects and was used in this study to manipulate viral titers of SINV-1 in individual *S. invicta* workers and whole *S. invicta* colonies. By exposing *S. invicta* workers and whole colonies to a whole isolated SINV-1 virions and double stranded RNA (dsRNA) complementary to SINV-1, viral titers of SINV-1 were shown to significantly increase and decrease respectively. Increases and decreases in SINV-1 viral titers were shown to correlate with *S. invicta* worker mortality in infected *S. invicta* colonies. Differences in gene expression across SINV-1 and dsRNA treated *S. invicta* workers was measured by predicting the existence of 110 micro RNAs (miRNAs) in *S. invicta*, and measuring the relative abundance of each. The expression of five screened miRNAs was measured in SINV-1 infected *S. invicta* workers exposed to SINV-1 viral isolate, water, and dsRNA, with miRNAs Bantam and miR-8 determined to be significant components of the innate immunity of *S. invicta* against SINV-1.

Chapter One

Literature Review

Characteristics of Red Imported Fire Ants

Insecta is a class of invertebrates within arthropoda that are comprised of a chitinous exoskeleton, compound eyes, three segment body (head, thorax, abdomen), antennae, and three pairs of jointed legs (Borror et al., 1989). There are over a million described species of insects, making them the most diverse group of animals on the planet and representative of over half of all known living organisms (Chapman, 2009). Many insect species are considered pests as they may transmit pathogens, damage agricultural goods and equipment, and can employ potentially deadly chemical defenses (Bale et al., 2008). Ants, members of the order Hymenoptera (Figure 1 below), are among the most widespread and damaging insects in many terrestrial ecosystems as they are highly adaptable to new environments (Wilson, 1971).

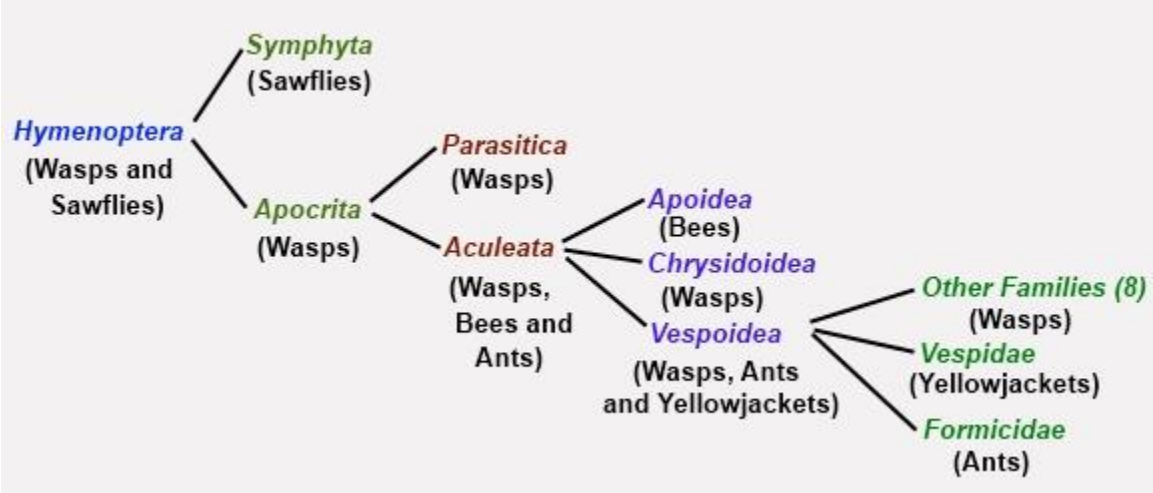

Figure 1**.** Phylogenetic Tree of the Order Hymenoptera Courtesy of http://askanaturalist.com

Solenopsis invicta Buren (red imported fire ant) was first observed in the United States between the 1930s and 1940's near Mobile, Alabama (Tschinkel, 2006). *S. invicta* infestations

in the United States have to spread more than 128 million hectares as of 2001 (Williams et al., 2001). Current control methods used to mitigate, repair, and prevent damage caused by *S. invicta* exceeds 6 billion dollars (U.S.) annually (Periera, 2003). Damage caused by *S. invicta* includes feeding on crops, such as, okra, corn, soybeans, potato, and peanuts (Adams, 1986), disturbance of urban electrical equipment for nest building (Coplin, 1989), and the threat of severe stings to humans and livestock. As recent as September 13, 2013, a 13 year old boy from Corpus Christi, Texas, died as the result of an allergic reaction caused by *S. invicta* stings (ABC news, 2013).

Invasive ant species establish outside their native ranges as a direct result of anthropogenic-mediated transportation (Williams, 1994, & McGlynn, 1999). Once established, invasive ant species outcompete and displace native species (McGlynn, 1999). Common traits associated with successful invasive ant species include relatively low dietary and nesting requirements, polygyny, colony budding, and reduced intraspecific aggression (Holoway et al., 2002). Polygyny has attributed to increased colony growth and budding, and is common to almost every invasive ant species with the rare exception of the monogyne form on *S. invicta* (Vargo et al., 1989). In monogyne *S. invicta* colonies, reproduction occurs via the dispersal of winged queens, each of which found their own colony (Markin et al., 1972). In polyginous species, budding of a new colony is accomplished by a gravid queen and a group of workers leaving the natal colony to establish a nearby colony (Wilson, 1971).

While both forms of *Solenopsis invicta* (monogynous and polygynous) are found in the United States, the poygynous form is of interest for two reasons. First, this form was once thought to be more economically and ecologically destructive, displacing native and other invasive ant species (Porter & Savignano, 1990). However, more recent studies have been used

to show that *S. invicta* does not significantly repress native ant populations (King & Tschinkel, 2006; Morrison, 2002). Second, the polygynous form of *S. invicta* was detected in the United States 20 years after the monogynous form was first reported (Glancey et al., 1973). Therefore, polygyny may have arisen in introduced populations in the United States, or more likely, that there was a secondary introduction of *S. invicta* (Porter et at., 1988). Emlen (1982) suggests that the polygyny arose in *S. invicta* due to pressure from predators and parasites. This in turn would have increased colony densities, causing a selective pressure for elevated nest-mate recognition abilities, promoting polygyny (Ross & Keller, 1995). However, genetic differences discovered between the two forms suggest that it is unlikely that polygyny arose from ecological constraints alone (Ross, 1997).

The range, mound size, and mound density of *S. invicta* in the southern United States has been shown to be greater when compared to ranges in native Brazil (Allen et al., 1974; Williams & Whitcomb, 1974; Fowler et al., 1990; and Porter et al., 1992). In the United States, *S. invicta* infestation covers more than 129 million hectares across 13 states and Puerto Rico (Callcott, 2002), while in Brazil, the native range spans approximately 30 thousand hectares (Tschinkel, 2006). Northern expansion of *S. invicta* in the Southern United States is most likely halted by harsher winters, while western expansion is halted by dessert conditions (Jouvenaz et al., 1977).

The success of *S. invicta* in the United States may be attributed to a lack of native control agents (Beckham, 1980), and the species ability to outcompete a majority of native ant species (Porter et al., 1992). *S. invicta* colonies in the United States are significantly greater in density, mound volume, and comprise a larger portion of the ant community when compared to the same fire ant species in their native range in Brazil (Porter et al., 1992, 1997). As of 2004, the list of known self-sustaining biological control agents for *S. invicta* in the U.S. included two species of

endoplasmic fungi (Jouvenaz & Kimbrough, 1991; Periera, 2004), a strepsipteran parasite (Kathirithamby & Johnston, 2001), and phorid flies of the genus *Pseudacteon* (Porter, 1998).

Disistroviridae: *Solenopsis invicta* Virus

The *Solenopsis invicta* virus (SINV), first described in 2004 (Valles et al., 2004) is a member of the family *Dicistroviridae*, classified as Group IV insect-infecting, positive sense, single-stranded RNA (Mattenleiter & Soborino, 2008), with a viral capsid surface protein derived of four groups of equilateral triangles (Rueckert, 1991). SINV is of the same family that is a contributor to honey bee mortality (Figure 2), and is thought to produce a similar effect in ants (Cox-Foster et al., 2007). Some insects infected with dicitroviruses display asymptomatically until stressed by an infection, environmental conditions, malnutrition, or other pathogens that can transform the virus into a lethal state (Christian & Scotti, 1998). Therefore, a dicistrovirus used in tandem with another stressor, such as a fungi or toxin, environmental distress, or immune gene knock-down may promote colony collapse when used as a management technique against *S. invicta.*

SINV-1 has been detected in every state in the USA where *S. invicta* is present with the exception of New Mexico (Valles, 2011), with infection rates ranging anywhere from <10% (Valles et al., 2007), and up to >90% of the total population (Valles et al., 2010). To date, every developmental stage in *S. invicta* including eggs and alates have screened positive for SINV-1, suggesting both horizontal as well as vertical viral transmission (Jouvenaz & Kimbrough, 1991; Hashimoto & Valles 2007; Hashimoto et al., 2007), with larvae and workers exhibiting the highest viral titers per individual (Hashimoto et al., 2007). A positive link has been observed between intra-colony prevalence of SINV-1, and viral titers of individual workers in the colony such that *S. invicta* colonies with greater SINV-1 infection rates also exhibited individuals with

greater viral loads (Valles et al., 2004). Prevalence of SIN-1 infection among wild colonies of *S. invicta* has been shown to be linked with seasonal temperature changes (Hertz & Thompson, 2011; Valles et al., 2010). During the warmer months, SINV-1 infection rates are likely elevated as a result of increased IGR IRES activity (Hertz & Thompson, 2011), enhancing ribosome domain activity, and augmenting viral replication.

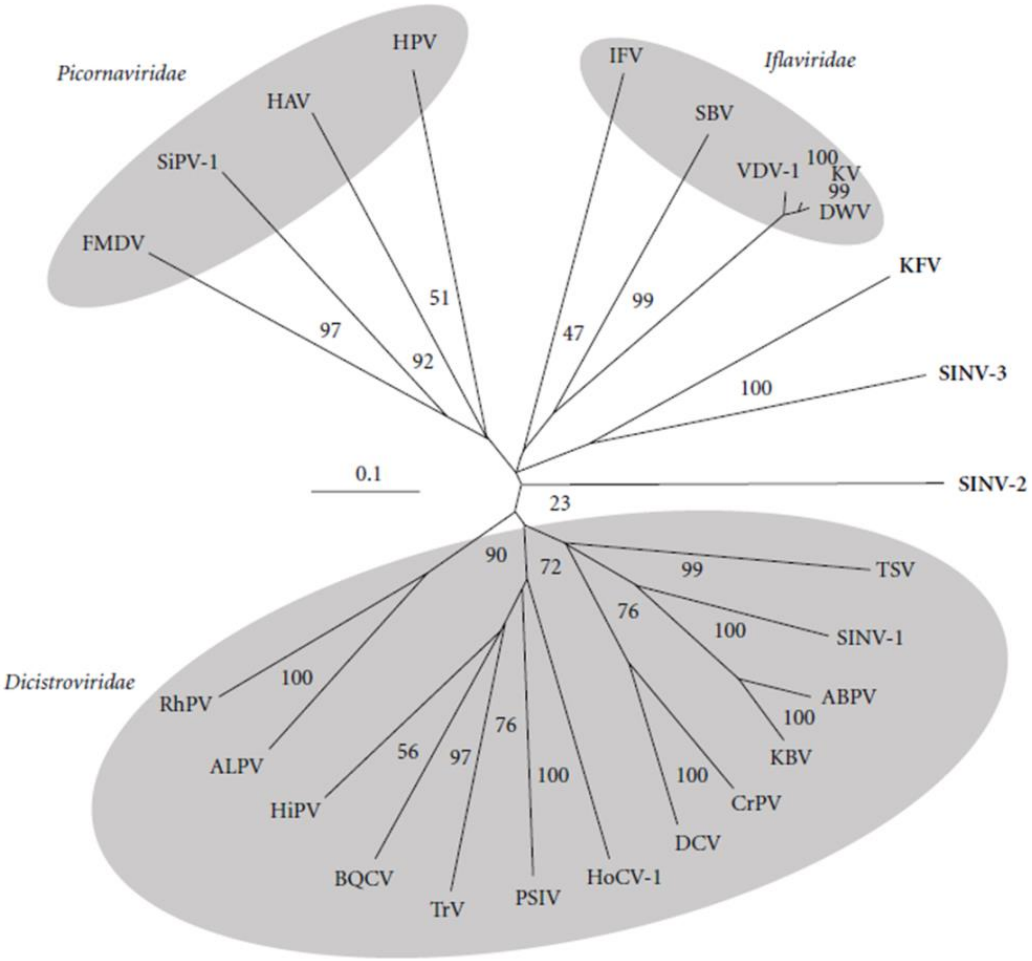

Figure 2**:** Phylogenetic Tree of SINV-1 as it Relates of other Members of Dicistroviridae such as Acute bee paralysis virus (ABPV) and Cricket paralysis virus (CrPV)(Valles, 2011)

The first dicitrovirus isolated from *S. invicta* (SINV-1) has an RNA genome of 8026 nucleotides long, a polyadenylated tail, is single stranded, is monopartite, and encodes two relatively large non-overlapping reading frames (Valles et al., 2004; 2007) (Figure 3 below). SINV-1 has a long untranslated region at the 5' end associated with translation, virulence, and encapsulation consistent with other dicistoviruses (Mettenleiter & Sobrino, 2008). Although virulence of ssRNA in ant species is relatively benign (Valles et al., 2004), by altering the amino acid composition, it may be possible to increase infectivity, pathogenicity, or transmissibility (Tufts et al., 2010). All three forms of SINV share the ability to infect all caste members among every developmental state, monogyne, and polygyne colony forms, horizontally (orally) between individuals, and vertically by transovum (Valles & Strong, 2005; Gomirez-Zilber & Thomas-Orillard 1993). Alternatively, viral particles may spread via infected feces, and from midgut tissue transmitted by trophallaxis (Hashimoto & Valles, 2007). A summary of this interaction can be seen below in figure 4.

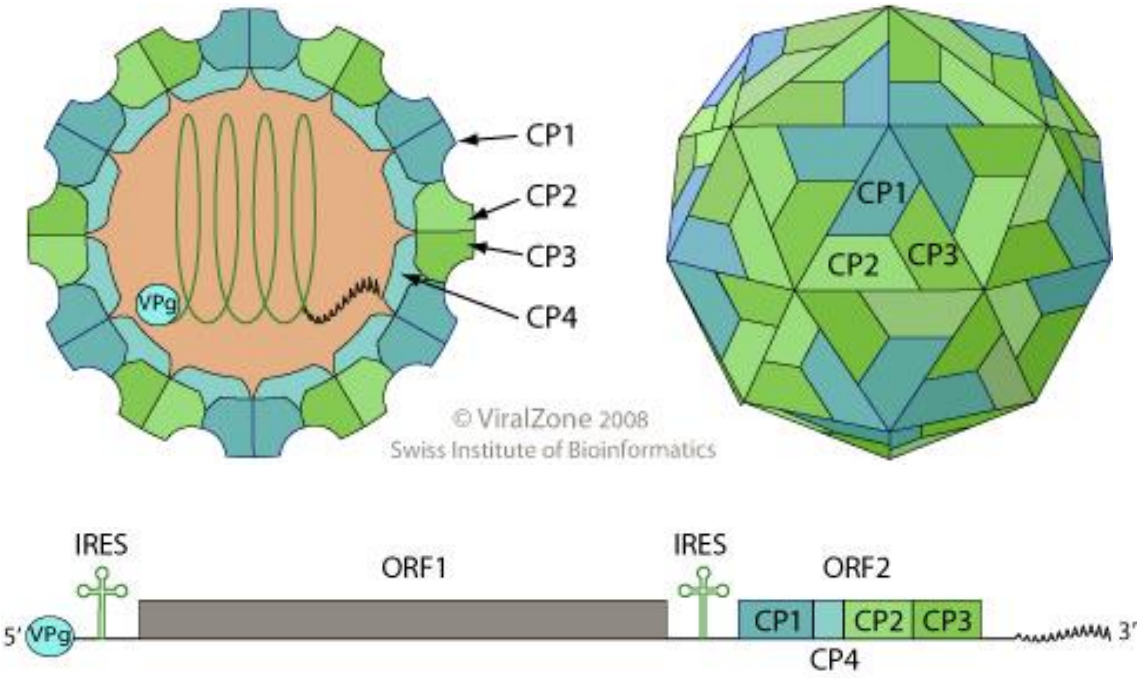

Figure 3. Gene map of Dicistroviridae courtesy of viralzone.org

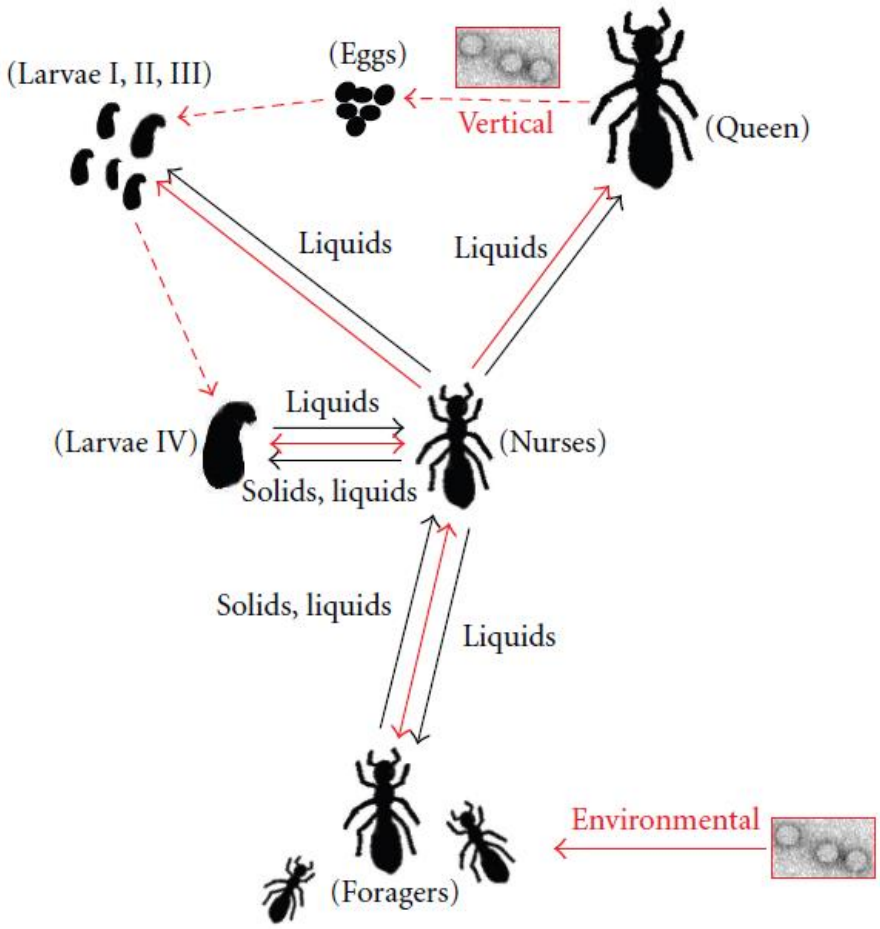

Figure 4. Routs of possible SINV-1 exposure in *S. invicta* colonies

Solenopsis invicta Control Methods

As of 2004, the list of known natural enemies for *S. invicta* in the U.S. included two species of endoparasitic fungi (Jouvenaz & Kimbrough, 1991; Periera, 2004), a strepsipteran parasite (Kathirithamby & Johnston, 2001), a microsporidian obligate parasite (Williams et al., 1998), a neogregarine parasite (Periera et al., 2002) and phorid flies of the genus *Pseudacteon* (Porter, 1998). Due to the lack of natural enemies against *S. invicta*, pest control experts have had to rely on insecticidal baits, which are an effective control method, but which pose downsides for long term use. Bifenthin, a known GABA disruptor, and hydramethylnon, a

metabolic inhibitor, are the primary ingredient in some fire ant baits and have been shown to be toxic to non-target insects, and degrade in wet conditions (El Hassani et al., 2005; Lucas & Invest, 1993). Therefore, a targeted, long-term, and self-regulating biological control agent for *S. invicta* may be a more effective alternative than currently used chemical control agents, possibly allowing for management of *S. invicta* populations on a relatively larger scale.

RNA interference (RNAi) is an emerging pest management technique that uses the introduction of novel, targeted double stranded RNA (dsRNA) sequences into pest organisms to degrade messenger RNA (mRNA) complementary to the sequences in the dsRNA (Mello & Conte, 2004). RNAi has been shown to knock down phenotypic expression of the PBAN, a gene that acts on sex pheromone production, and increase mortality in *S. invicta* larvae (Choi et al., 2012). *S. invicta* individuals treated with dsRNA complementary to sequences of PBAN have been shown to produce less trail pheromone, which may have application as a possible control method (Choi et al., 2012).

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Chapter Two

The Down-Regulation of the Solenopsis invicta Virus-1 with RNA interference

1. Introduction

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera, Formicidae), was first observed in the United States between the 1930s and 1940s near Mobile, Alabama (Tschinkel, 2006); since then, *S. invicta* populations in the United States have spread to more than 128 million hectares as of 2001 (Williams et al., 2001). Current management techniques used to mitigate, repair, and prevent damage caused by *S. invicta* exceeds 6 billion dollars (U.S.) annually (Piera, 2003). Damage caused by *S. invicta* includes feeding on crops, such as, okra, corn, soybeans, potato, and peanuts (Adams, 1986), disturbance of urban electrical equipment for nest building (Coplin, 1989), and the threat of severe stings to humans and livestock. *S. invicta* has also been implemented in the displacement of native species in the United States including other species of ants (Wilson, 1951; Tennant & Porter, 1991), as well as mammals (Laakkonen et al., 2001) birds (Feare, 1999), and lizards (Jourdan et al., 2001).

The success of *S. invicta* in the United States may be attributed to a lack of native control agents (Beckham, 1980), and *S. invicta*'s ability to outcompete a majority of native ant species (Porter et al., 1992). *S. invicta* colonies in the United States are significantly greater in density, mound volume, and comprise a larger portion of the ant community when compared to the same fire ant species in their native range in Brazil (Porter et al., 1992, 1997). As of 2004, the list of known natural enemies for *S. invicta* in the U.S. included two species of endoparasitic fungi (Jouvenaz and Kimbrough, 1991; Periera, 2004), a strepsipteran parasite (Kathirithamby & Johnston, 2001), a microsporidian obligate parasite (Williams et al., 1998), a neogregarine parasite (Periera et al., 2002) and phorid flies of the genus *Pseudacteon* (Porter, 1998). Due to the lack of natural enemies against *S. invicta*, pest control experts have had to rely on insecticidal

baits, which are an effective control method, but which pose downsides for long term use. Bifenthin, a known GABA disruptor, and hydramethylnon, a metabolic inhibitor, are the primary ingredient in some fire ant baits and have been shown to be toxic to non-target insects, and degrade in wet conditions (El Hassani et al., 2005; Lucas & Invest, 1993). Therefore, a targeted, long-term, and self-regulating biological control agent for *S. invicta* may be a more effective alternative than currently used chemical control agents, possibly allowing for management of *S. invicta* populations on a relatively larger scale.

The *Solenopsis invicta* virus (SINV), first described in 2004 (Valles et al., 2004), is a member of the family *Dicistroviridae*, classified as Group IV insect-infecting, positive-sense, single-stranded RNA virus (Mattenleiter & Soborino, 2008). SINV is a member of the same family that is associated with honey bee mortality, is only known to infect *Solenopsis sp.*(Valles et al., 2007), and may produce similar mortality in *S. invicta* as it does in honey bees (Cox-Foster et al., 2007). Some insects infected with dicitroviruses have latent infections which appear asymptomatic until stressed by a secondary infection, environmental conditions, malnutrition, colony re-location into the laboratory, or other pathogens that can transform the virus into a patent infection which displays a lethal state (Christian & Scotti, 1998). The first dicitrovirus isolated from *S. invicta* (SINV-1) has an RNA genome of 8026 nucleotides long, a polyadenylated tail, is single-stranded, monopartite, and encodes two relatively large non-overlapping reading frames, which causes dicistroviruses like SINV-1 to replicate in a messenger RNA (mRNA) like manner (Valles et al., 2004; 2007). Currently, three strains of SINV have been described, namely SINV-1, SINV-2 (Hashimoto & Valles, 2008), and SINV-3 (Valles & Hashimoto, 2009), as well as a sub-strain of SINV-1, SINV-1 (TX-5) (Tufts et al., 2010). Both SINV-1 and SINV-3 have been successfully transmitted to uninfected *S. invicta* colonies in

laboratory conditions and produce sustained colony infection (Tufts et al., 2011)(Valles et al., 2013).

SINV-1 has been detected in every state in the USA where *S. invicta* is present with the exception of New Mexico (Valles, 2011), with infection rates ranging anywhere from <10% (Valles et al., 2007), and up to >90% of the total population (Valles et al., 2010). To date, every developmental stage in *S. invicta* including eggs and alates have screened positive for SINV-1, suggesting both horizontal as well as vertical viral transmission (Jouvenaz & Kimbrough, 1991; Hashimoto & Valles 2007; Hashimoto et al., 2007), with larvae and workers exhibiting the highest viral titers per individual (Hashimoto et al., 2007). A positive link has been observed between intra-colony prevalence of SINV-1, and viral titers of individual workers in the colony such that *S. invicta* colonies with greater SINV-1 infection rates also exhibited individuals with greater viral loads (Valles et al., 2004). Prevalence of SIN-1 infection among wild colonies of *S. invicta* has been shown to be linked with seasonal temperature changes (Hertz & Thompson, 2011; Valles et al., 2010). During the warmer months, SINV-1 infection rates are likely elevated as a result of increased IGR IRES activity (Hertz & Thompson, 2011), enhancing ribosome domain activity, and augmenting viral replication. Therefore, the time in which colonies are collected as related to seasonal temperature must be considered when performing studies related to SINV-1 when locating uninfected *S. invica* colonies may become more difficult.

RNA interference (RNAi) is an emerging pest management technique that uses the introduction of novel, targeted double stranded RNA (dsRNA) sequences into pest organisms to degrade messenger RNA (mRNA) complementary to the sequences in the dsRNA (Mello & Conte, 2004). RNAi has been shown to knock down phenotypic expression of the PBAN, a gene that acts on sex pheromone production, and increase mortality in *S. invicta* larvae (Choi et al.,

2012). *S. invicta* larvae that were indirectly fed a dsRNA construct complementary to sequences of PBAN have been shown to have significantly increased mortality, while injection of these same dsRNA constructs successfully knocked down PBAN gene expression (Choi et al., 2012). Gene knock-down was not noted in adult *S. invicta* workers fed the dsRNA construct (Choi et al., 2012), it is likely that the mRNA-like manner in which SINV-1 replicates may make it a possible target for degradation via the RNAi pathway. In this study, RNAi and whole isolated virions were used as a tool to manipulate viral titers of SINV-1 by feeding dsRNA designed to the SINV-1 viral capsid gene.

2. Materials and Methods

2.1. Colony collection and Maintenance

S. invicta colonies were collected from two sites in August, 2013 from Smith County, Texas. Approximately 5,000 individuals (workers, brood and queens) were excavated from seven colonies and placed in five gallon containers. Three to five inches of the top of each container was coated with INSECT-a-SLIP™ (BioQuip Products, Rancho Dominguez, CA) to prevent ants from escaping. Adults and brood were separated from soil by dripping tap water (10ml/min) into the containers. After removal from containers, ants were placed in 29- by 15- by 11-cm clear plastic shoe boxes, which were coated with 2 to 3 inches of INSECT-a-SLIP. Smaller clear boxes (11- by 11- by 4-cm) coated with approximately 3mm Faststone™ (Atlantic Dental Supply, Durham, NC) dental plaster on the bottom, were placed in the larger colony containers to serve as the brood chamber. The dental stone base was saturated prior to introduction with autoclaved tap water to maintain humidity in brood chambers. Colonies were maintained in the lab (22.5 °C, 12 h L:D), and received a diet described by Dussutour and Simpson (2008) as needed, with unlimited water.

2.2. SINV-1 Viral RNA Extraction and Screening

S. invicta RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacture's protocol. Ten individual adult workers were used in each extraction, and three extractions were performed for each of the seven colonies collected. A One Step Reverse Transcriptase PCR (qRT-PCR) (Invitrogen, Carlsbad, CA) was used to produce cDNA complimentary to a sequence of the SINV-1 genome using primers designed by Valles and Bextine (2011) (F,5′-CATCGAGAT- CTATTGCTACCC-TTCCAAATGCA-3′, R, 5′-ACTTACCTACGCCACTTTCACCAAA- CAACCA-3′). The RNA RT-PCR was performed using the following thermal profile: 50°C for 10min, 95°C for 5min, denatured 95°C for 10 s, annealed 60°C for 30 s, repeated 40 times, followed by a melt curve obtained by ramping from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, then a hold at 4°C. Samples that were amplified by RT-PCR and produced a corresponding band length (approximately 148bp) were considered to be virally positive (1% agarose gel). Samples were not tested for the presence of SINV-2 or SINV-3.

2.3. Isolation of whole virus

Whole virus was extracted from SINV-1 infected *S. invicta* colonies using a method modified from Hunter et al. (2003). Approximately 5000 infected ants were homogenized in 50ml of 0.01 M phosphate buffer pH 7.2 containing 10mg of diethyl thio carbamic acid (DETCA) (Sigma-Aldrich, St. Louis, MO), then centrifuged for 20 min at 300xg. The supernatant was transferred and rotated at 124,500xg for four hours at 4°C, then discarded. The resulting pellet was dissolved with 750µl of 0.01 M phosphate buffer containing 0.4% sodium-deoxycholic acid (Sigma-Aldrich, St. Louis, MO) and 4% polyethylene glycol hexadecyl ether (Brij-52) (Sigma-Aldrich, St. Louis, MO), then centrifuged at 300xg for 15 min. The solution

was allowed to drip through a 0.45µ filter at 35°C, transferred to a dialysis membrane, and placed in nano-pure water at 4°C for approximately 5-6 hours. The precipitate inside of the dialysis tubing was collected and stored at -80°C. Approximately 1 ml of whole virus was extracted and virus concentrations of SINV-1 were confirmed with NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA), qRT-PCR, and gel electrophoresis.

2.4. Construction of dsRNA

The SINV-1 capsid gene dsRNA was synthesized from the total TRIzol (Life Technologies, Carlsbad, CA) RNA extraction from SINV-1 positive *S. invicta* individual workers using specific PCR primers (5′-TGGG- TGGCATAACGCTTGAT-3′ and 5′-CCAGTACTTGCATGGTCC-3′) in a qRT-PCR to amplify a 248-bp SINV-1 capsid gene cDNA fragment. Resulting PCR product was then run through a 2.5% agarose gel and the corresponding band was excised using a QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). A second qRT-PCR was used to amplify gel extracted SINV-1 cDNA fragment with specific 5′-T7-appended PCR primers (5′-TAATAC GAC- TCACTATAGGGTG- GGTGGCATAAC GCTT- GAT-3′ and TAATACGACTCA CTATA- GGGCCAGTACTTGCA- TGGTCC-3′). This fragment served as the template for dsRNA synthesis using the HIScribe T7 *In Vitro* Transcription Kit (New England BioLabs, Ipswich, MA).

2.5. Delivery of dsRNA

To deliver dsRNA, five adult *S. invicta* workers from a SINV-1 positive colony were placed into a Petri dish with Whatman filter paper cut into 1/8" pieces and fasted for one day. Filter paper pieces that were the Petri dish was saturated with purified dsRNA diluted in 100µl of autoclaved tap water at a concentration of 50ng/µl. This process was repeated simultaneously along with two other trials. First, control group exposed to 100ul of autoclaved tap water, and

second, 100µl of a 50ng/µl concentration of whole viral extract diluted in autoclaved tap water. Each treatment group was replicated five times. After five days, all 75 workers used in the trial were collected, sacrificed, and washed in 95% ethanol and autoclaved Nanopure tap water to ensure virus was not detected from the exterior of the individuals. Ants from each dish were collected in 1.5ml centrifuge tubes and subjected to Trizol RNA extraction (following manufacture's protocol). Samples were then screened for SINV-1 using qRT-PCR following the protocol described above.

2.6. Establishing a standard curve

The standard curve used in this study was produced by amplifying the sequence of SINV-1 using SINV-1 screening primers (described above) in RT-PCR, then running the PCR product through a 2.5% agarose gel and excising the band using a QIAquick Gel Extraction kit. The resulting amplicon was then amplified again using an identical PCR protocol to the SINV-1 screen, as well as an identical gel extraction procedure, then diluted in 10 sequential 1/100µl serial dilutions. The concentration of each dilution was determined using Nanodrop 1000, which was then used to calculate the total number of amplicon copies in in each dilution. Five amplicon dilutions were incorporated in q-PCR along with each treatment sample to serve as known standards. ANOVA and Tukey's post-hoc tests were used to determine significance difference in the number of viral transcripts.

3. Results

3.1. Detection of SINV-1, and isolation and delivery of whole virus

Out of the seven *S. invicta* colonies collected and screened for SINV-1, all had workers that tested positive for SINV-1. The primers used to screen for SINV-1 produced an amplicon of 148bp that was verified by PCR and gel electrophoresis. Several *S. invicta* colonies were

sacrificed and used to extract whole viral isolate and approximately 1-2ml of extract was recovered from each. Individuals treated with whole viral isolate had a significantly higher number of viral transcripts on average than did individuals from the same colony that were treated with dsRNA or water ($p < .05$) (Figure 15.

3.2. Construction and delivery of dsRNA-SINV

The primers used to synthesize dsRNA amplified a sequence of the SINV-1 genome 248bp in length that was confirmed using RT-PCR and gel electrophoresis (Figure 6). SINV-1 positive *S. invicta* workers exposed only to water, compared to *S. invicta* workers fed dsRNA-SINV had on average, a significantly lower number of SINV-1 viral transcripts ($p < 0.05$)(Figure 5).

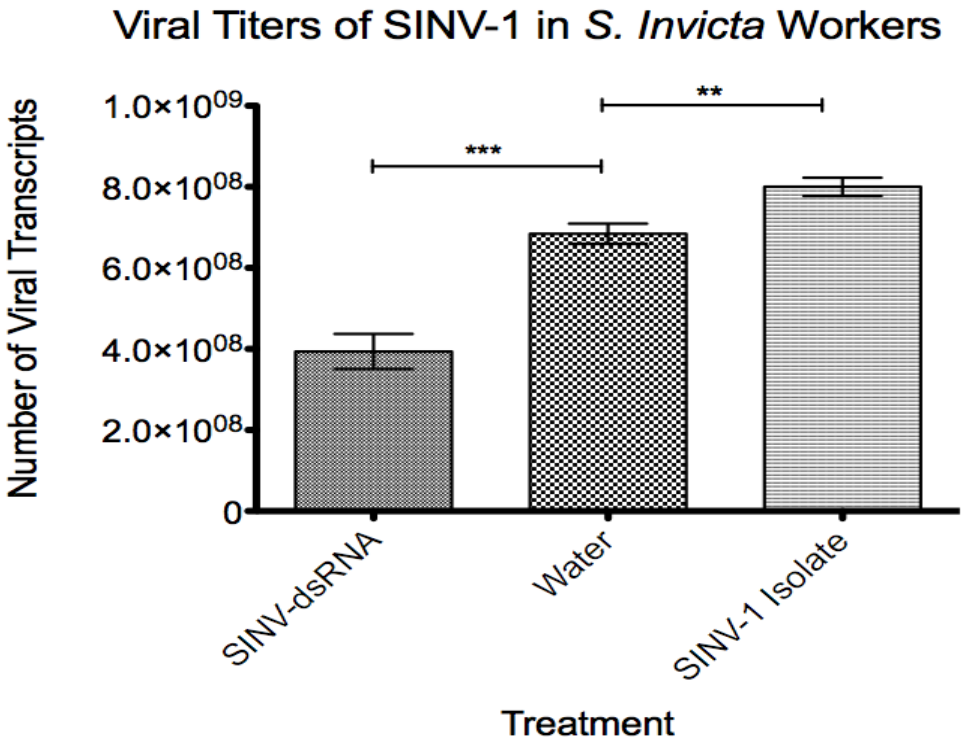

Figure 5**.** Abundance of SINV-1 viral transcripts between water control, dsRNA treated (***, $p<0.0005$), and SINV-1 treated (**, $p<0.005$), SINV-1 positive *S. invicta* workers. Error bars are based on mean and standard error (ANOVA).

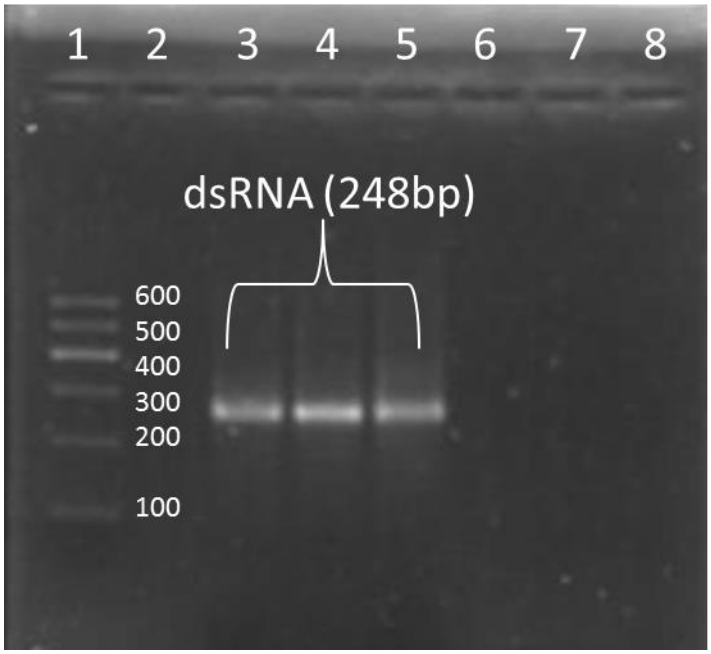

Figure 6**.** Confirmation of dsRNA using 2.5% agarose gel, and GelPilot 100bp Ladder (cat. No. 239035)

4. Discussion

4.1. Colony Collection and SINV-1 Screen

High prevalence of SINV-1 in colonies collected was likely due to the combination of sampling during peak temperatures in summer months (August), as well as a relatively low sample size (n=7). The endemic nature of SINV-1 in local populations presents two problems for researchers studying *S. invicta* in the laboratory. First, procuring a viral negative *S. invicta* colony to serve as a control becomes increasingly more difficult during the warmer spring and summer months, as viral replication is increased (Hertz & Thompson, 2011). Second, the process of removing and transporting *S. invicta* colonies from a field site to the laboratory is known to be a stressor that can trigger a change in asymptomatic SINV-1 infection, lead to brood death, and may ultimately cause colony collapse (Valles et al., 2004; Chen & Siede, 2007). The RNAi tools utilized in this study have potential use in alleviating both of the issues described above. In conditions where finding a viral negative colony are difficult, it may be possible to down-

regulate viral titers of SINV-1 at the colony level in the laboratory for use as control colonies in future studies. Finally, when colony collapse conditions occur in the laboratory, in previously asymptomatic infection becoming symptomatic due to the stress of transport from the field, it may be possible to use RNAi to reduce viral titers, mitigate brood and worker mortality, and prolong colony retention.

4.2. Delivery of dsRNA and SINV-1

To determine if viral titers of SINV-1 could be manipulated in laboratory colonies of *S. invicta* using RNAi, dsRNA coded for sequences of SINV-1 viral capsid was administered orally in a water solution to adult *S. invicta* workers. Thus, it was possible to significantly reduce SINV-1 viral transcript copy number using this technique. The mRNA-like structure and method of replication of SINV-1 may explain RNAi-mediated gene knock-down success.

4.3. Future implications

Reduction of viral titers of SINV-1 using an RNAi-based approach was successful in individual *S. invicta* workers under laboratory conditions. Evidence as to whether this can aid research colony production and health still need to be validated. The effect of RNAi-based viral gene knock-down on mortality was not addressed in this study and attempts should be made to investigate if the reduction in copies of SINV-1 viral transcript decreases overall colony mortality and extends colony retention in the laboratory. Furthermore, by understanding the mechanisms of SINV-1 infection and its role in *S. invicta* pathology may provide clues to how best use SINV type viruses as biopesticides, or biological control agents against *S. invicta.*

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Chapter Three

Manipulation of the Solenopsis invicta Virus-1 in *S. invicta* Colonies

1. Introduction

The red imported fire ant, *Solenopsis invicta* Buren, is an invasive ant species from South America that first arrived in the United States sometime in the 1930's or 1940's in Mobile Alabama (Tschinkel, 2006). As of 2001, *S. invicta* infestation has spread to approximately 128 million hectares across the southern United States, with attempts to mitigate, and control damage caused by *S. invicta* reaching approximately six billion dollars (U.S.) annually (Williams et al., 2001; Pereira, 2003). Approximately 3.6 billion dollars of the damage caused by *S, invicta* goes to the repair and replacement of structures in businesses, schools, government buildings and institutions with another 1.35 billion going to management of the *S. invicta* populations (Pereira, 2003). Currently, the list of known natural enemies for *S. invicta* in the United States is relatively low when compared to the species native range. In native Brazil, approximately 30 different species exists which compete with and control *S. invicta* populations; while in the United States, known natural enemies include the Tawny Crazy ant (*Nylanderia fulva*) (Chen et al., 2013), a microsporidian obligate parasite (Williams et al., 1998), a neogregarine parasite (Pereira al., 2002), a strepsipteran parasite (Kathirthamby & Johnston, 2001), two species of endoparasitic fungi (Jouvenaz & Kimbrough, 1991; Pereira, 2004), phorid flies (*Pseudacteon*) (Porter, 1998), and three RNA viruses of the family dicitroviridae (Valles & Hashimoto, 2009).

Dicistroviridae, formerly known as the "Cricket paralysis-like viruses," is a class IV insect-infecting viruses that were discovered to be significant contributors of honey bee mortality (Bailey and Woods, 1977), and have been isolated from six invertebrate orders of Insecta and decapod crustaceans (Lightner, 1996; Lightner et al., 1997). One example, the Cricket paralysis

virus (CrPV) is a widely distributed virus (Reinganum et al., 1981), with the widest range of any invertebrate small RNA virus (Christian and Scotti, 1998). Diseases caused by dicitroviridaes reduce longevity and fecundity in infected individuals and in some cases may lead to a relatively rapid onset of paralysis (Hatfill & Williamson, 1990). CrPV in particular has been used in the management of pest populations, such as the olive fruit fly, *Bactrocera oleae* (Manoussis & Moore, 1987). The utilization of such a virus for use as a management technique against *S. invicta* would prove to be economically significant as a long term, self-sustaining, biological control agent.

The *Solenopsis invicta* virus-1 (SINV-1), discovered in 2004 (Valles et al., 2004), is a single stranded, positive sense, monopartite, poly-addenelated, picorna-like member of the family Dicistroviridae (Bonning, 2009). Two other strains, SINV-2 and SINV-3, were discovered in 2008 and 2009, respectively. Phylogenetic analysis has been used to suggest that SINV-1 likely arrived in North America from the introduction of *S. invicta* as there is a divergence between isolates infecting South America (Allen et al., 2010). Relatively little is known of the pathogenicity and mechanism of SINV-1 as infection remains asymptomatic until infected individuals are secondarily stressed before SINV-1 is transformed into a lethal state (Christin and Scotti, 1998). While SINV-1 infection has been associated with *S. invicta* larval mortality of laboratory colonies, no overt symptoms are present in field colonies (Valles et al., 2004).Even with the absence of notable symptoms of SINV-1 infected *S. invicta* individuals in the field, infected *S. invicta* have been shown to performed significantly worse when competing against other ant species than did non-infected *S. invicta* (Chen et al., 2011), suggesting that SINV-1 may ultimately have a negative impact on *S. invicta* fitness.

SINV-1 viral expression is strongly correlated to seasonal temperature, and care must be taken when colonies are collected as the warmer the season, as the higher the rates of SINV-1 infection tend occur (Valles et al., 2010). This temperature dependent effect is possibly due to more efficient IGR IRES activity, which displays up to a 3-5 fold increase in activity in higher temperatures (Jang & Eric, 2010). Titers of SINV-1 have been detected in all *S. invicta* caste member (Hashimoto & Valles, 2007), with viral concentrations peaking in the midgut of adults and the alimentary canal of larval stages (Miranda et al., 2010). Transmission of SINV-1 occurs not only through trophallaxis or substrate contamination via defecation, but vertically as well from queen to eggs (Hashimoto & Valles, 2007). The highest viral loads across a SINV-1 infected *S. invicta* colonies are found in the larvae and workers, ranging from 10^8 to 10^9 viral copies per individual (Hashimoto et al., 2007), and a strong positive relationship can be observed between the SINV-1 viral titer in individual ants and intracolony prevalence of SINV-1 (Hashimoto et al., 2007). Through manipulation of viral titers, it may be possible to utilize SINV-1 as a potential long term, self-replicating biological control agent against *S. invicta.*

RNA interference (RNAi) is a known regulator of biological systems in insect species, and may have potential as a means to regulate viral titers of SINV-1 in SINV-1 infected *S. invicta* individuals. Through the introduction of double-stranded RNA (dsRNA), messenger RNA (mRNA) sequences complementary to those found in the dsRNA can be degraded. Double-stranded RNA has been used to down-regulate two genes in the pest termite *Reticulitermes flavipes,* an endogenous digestive cellulase enzyme and a caste-regulatory hexmerin storage protein, via voluntarily feeding adult *R. flavipes* dsRNA constructs (Zhou et al., 2008). In another study, the indirect feeding of dsRNA complementary to sequences in the gene PBAN in *S. invicta* larvae was shown to significantly increase mortality, while injection of these same

dsRNA sequences were shown to knock down PBAN gene expression (Choi et al., 2012). The mRNA like structure of SINV-1 may make it a possible target for down-regulation via the use of RNAi technology. In this study, whole viral isolate and novel dsRNA sequences complementary to SINV-1 viral capsid genes were voluntarily fed to adult *S. invicta* workers in SINV-1 negative *S. invicta* colonies to first increase, then decrease viral titers of SINV-1 over a 30 day period. The number of dead adult *S. invicta* workers was also tracked over the 30 day period to determine if a correlation exists between viral titers of SINV-1 and worker mortality.

2. Materials and Methods

2.1 Colony collection and Maintenance

S. invicta colonies were collected from sites in Angelina County, Texas during the month of July, 2014. Approximately 10,000 individuals, comprised of workers, brood, and queens were excavated from 16 colonies and placed in five gallon containers. To prevent the ants from escaping, the top three inches of each container was coated with INSECT-a-SLIP™ (BioQuip Products, Rancho Dominguez, CA). Adults and brood were separated from the containers by dripping approximately 10ml of tap water every minute until they floated to the surface, and then removed and placed in 29- by 15- by 11-cm plastic shoe boxes with the top two inches coated with INSECT-a-SLIP. A second, smaller box (11- by 11- by 4-cm), with the bottom coated in 3mm Fastone™ (Atlantic Dental Supply, Durham, NC) dental plaster, was placed in the larger colony boxes to serve as the brood chamber. The dental stone was saturated with autoclaved tap water before introduction of the colony to maintain humidity in the brood chamber. Colonies were maintained in the laboratory (22.5C, 12 h L:D), and were fed a diet modified from Dussutour and Simpson (2008) as needed, with unlimited water.

2.2 SINV-1 Viral RNA Extraction and Screening

To screen for the presence of SINV-1, ten individual workers were removed at random from each colony, and each had total RNA extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) as per the manufacture's protocol. Three RNA extractions and viral screens were performed for each colony. A One Step Reverse Transcriptase PCR (qRT-PCR)(Invitrogen, Carlsbad, CA) was along with primers designed by Valles and Bextine (2011) (F,5′-CATCGA-GATCTATTGCTACCCTTCCAAATGCA-3′, R, 5′-ACTTACCTACGCCACTTTCACCA-AACAACCA-3′) to produce cDNA complementary to a sequence of the SINV-1 genome. The RNA RT-PCR was performed using the following thermal profile: 50°C for 10min, 95°C for 5 min, denatured at 95°C for 10s, annealed at 60°C for 30s, cycled 40 times, followed by a melt curve obtained from increasing the temperature from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, then a hold at 4°C. Template cDNA that was amplified by RT-PCR that produced an amplicon of 148bp was considered to be positive for SINV-1. Colonies were not screened for the presence of SINV-2 or SINV-3.

2.3 Isolation of whole Virus

Whole SINV-1 viral particles were collected from SINV-1 positive *S. invicta* colonies using a method modified from Hunter et al. (2003). Approximately 5,000-10,000 individual ants from an infected colony were homogenized in 50ml of 0.01M phosphate buffer pH 7.2 containing 10mg of diethyl thio carbamic acid (DETCA) (Sigma-Aldrich, St. Louis, MO), then centrifuged at 300xg for 20min. The supernatant was transferred and centrifuged for four hours at 124,500xg and 4°C, then discarded while the resulting pellet was dissolved in 750µl of 0.01 M phosphate buffer containing 0.4% sodium-deoxycholic acid (Sigma-Aldrich, St. Louis, MO), then rotated for 15 min at 300xg. This solution was allowed to drip through a 0.45µ filter at

35°C, then transferred to a dialysis membrane (Spectrum Laboratories inc., Rancho Domniguez, CA), and placed in nano-pure water at 4°C for approximately 5-6 hours. The precipitate formed inside the dialysis membrane was collected and stored at -80°C. Viral concentrations of SINV-1 were confirmed through TRI-zol RNA extraction, RT-PCR, and gel electrophoresis.

2.4 Construction of dsRNA

The dsRNA constructed for this study was designed from primers coded for a SINV-1 viral capsid gene (5′-TGGGTGGCATAACGCTTGAT-3′ and 5′-CCAGTACTTGCATGGTCC-3′), and synthesized from TRIzol extracted RNA from SINV-1 positive *S. invicta* individuals. A qRT-PCR was used to amplify the viral capsid sequence, and the resulting PCR product was then run through a 2.5% agarose gel and the corresponding band was excised using a QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). A second PCR was used to amplify the target gel extracted SINV-1 viral capsid cDNA fragment using specific 5′-T7-appended primers (5′-TAATACGACTCACTATAGGGTGGTGGCATAACGCTTGAT-3′ and 5′-TAATACGACTCACTATAGGGCCAGTACTTGCATGGTCC-3′), which served as the template for dsRNA synthesis using the HIScribe T7 *In Vitro* Transcription Kit (New England BioLabs, Ipswich, MA).

2.5 Establishing a Standard Curve

A standard curve used to quantify viral titers of SINV-1 in qRT-PCR was designed by amplifying a sequence of SINV-1 with the SINV-1 specific screening primers (described above) in RT-PCR, then running the PCR product through a 2.5% agarose gel and excising the band using a QIAquick Gel Extraction kit. The extracted amplicon was used as the template for a second, identical PCR using the same SINV-1 screening primers as before, followed by an identical gel extraction procedure, then diluted in ten sequential 1/100µl serial dilutions. The

concentration of each dilution was determined using NanoSpec 1000 (ThermoScientific, Wilmington, DE), which was then used to calculate the total number of viral amplicon copies present in each dilution. Five of these dilutions were incorporated into each q-PCR along with the unknown samples to serve as known standards.

2.7 Delivery of Whole Virus, dsRNA, and Viral Screening

Eight SINV-1 viral negative colonies were randomly separated into one of two treatment groups. The first group was exposed to whole isolated SINV-1 and the second was exposed to a water control. *S.* invicta colonies were exposed to SINV-1 by incorporating 5ug of whole viral isolate into the five grams of food given to each colony. Worker mortality for each colony was tracked over a thirty day period by collecting dead *S. invicta* workers that were deposited into trash piles by live workers. After 15 days, SINV-1 exposed *S. invicta* colonies were exposed to dsRNA complementary to SINV-1 viral capsid by incorporating 5ug of purified dsRNA into the five grams of food given to each colony. To determine changes in SINV-1 viral expression over the thirty day period, five random *S. invicta* workers were collected from each colony on day one, 15, and 30, and were used in a Trizol RNA extraction, followed by qRT-PCR using the SINV-1 screening primers and protocol described above to measure levels of SINV-1 viral transcript in each colony. This screening process was replicated three times for each colony, with significance in viral titers determined using repeated measures ANOVA. Daily mortality for each *S. invicta* colony was assessed by collecting the dead adults that were carried to the trash piles surrounding the colony.

3. Results

3.1 Detection of SINV-1, isolation of whole SINV-1, and construction of dsRNA

Out of the 16 *S. invicta* colonies collected and screened, six tested positive for SINV-1. The primers used to amplify sequences of SINV-1 for screening produced an amplicon of 148bp that was verified by PCR and gel electrophoresis. Three SINV-1 infected colonies were sacrificed and used to extract approximately 1-2ml of whole viral isolate from each. The concentrations of each were verified using NanoSpec 1000, and were determined to be approximately 80-100ng/µl. The primers used to design the dsRNA produced an amplicon of 248bp, which was verified using gel electrophoresis.

3.2 *S. invicta* worker mortality and SINV-1 Viral Titers

Cumulative adult *S. invicta* worker morality was determined to be significantly higher on average in SINV-1 treated colonies over the 30 day period when compared to the control colonies ($p < 0.05$) (Figure 7), with a significant increases in mortality on days 11 ($p < 0.05$), 13 ($p < 0.05$), 14 ($p < 0.05$), and 15 ($p < 0.05$). During the first 11 days of infection, cumulative mortality between both treated and control colonies were not significantly different, but after day 11, *S. invicta* colonies exposed to SINV-1 were shown to have significantly higher worker mortality than control colonies ($p < 0.05$). Worker mortality after treatment with dsRNA in SINV-1 positive colonies (denoted by a vertical line on day 15) was not significantly different that worker mortality in uninfected colonies (Figure 7). Viral titers of SINV-1 were significantly higher in SINV-1 exposed *S. invicta* colonies after 15 days than uninfected *S. invicta* colonies ($p<0.05$) (Figure 8). At the 30 day mark, viral titers of SINV-1 had decreased significantly when compared to viral titers at day 15 ($p<0.05$) (Figure 8), but remained significantly higher than control SINV-1 viral titers ($p<0.05$). In unexposed control colony, measured background levels

of virus remained constant over the 30 day period. Increases and decreases in SINV-1 viral titers were shown to directly correlate with increases and decreases in mortality ($p<0.05$).

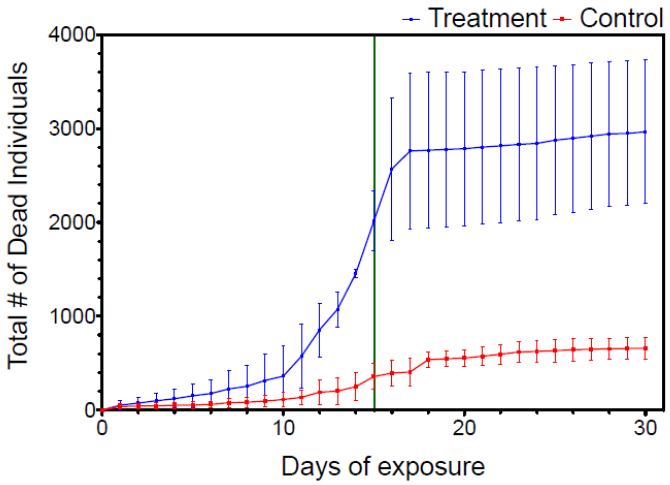

Figure 7**.** Cumulative mortality between SINV-1 exposed (treatment), and untreated (control) *S. invicta* colonies over a 30 day period. Day 15 SINV-dsRNA treatment is denoted by the vertical line. Standard error is represented for each day and significance was determined using ANOVA.

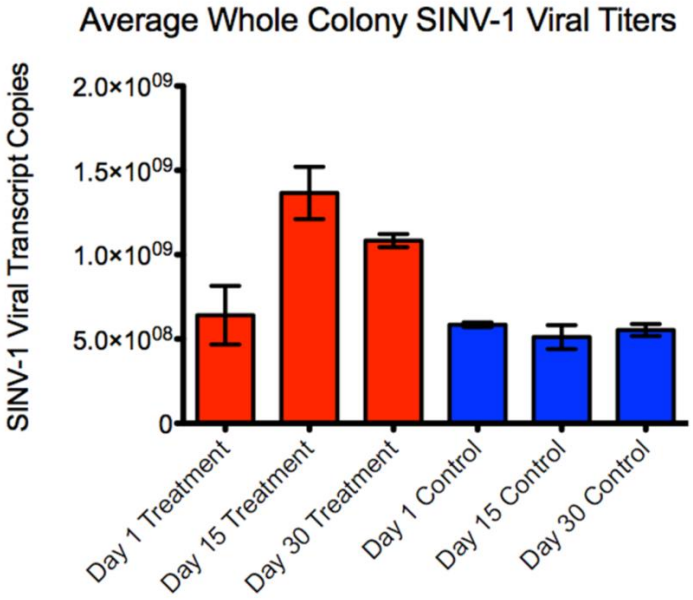

Figure 8**.** Viral titers of SINV-1 compared between days 1, 15, and 30 for SINV-1 exposed (treatment), and untreated (control). Treated groups were exposed to SINV-dsRNA on day 15. Standard error is represented on each graph and significance was determined using repeated measures ANOVA.

4. Discussion

4.1 Delivery of Whole SINV-1 Isolate and the Effect on *S. invicta* Worker Mortality

While the presence of absence of SINV-1 did correlate to mortality rates, it is not understood if mortality was due to a direct effect on worker fitness, or if SINV-1 secondarily transmitted to the brood by workers was increasing larval mortality, thereby leading to starvation of the workers who rely on the brood to feed. However, obtaining accurate brood counts can be difficult as deceased larvae are often cannibalized by adult workers (Holldobler and Wilson, 1990); therefore, reducing the accuracy of mortality counts. SINV-1 infected queens may have produced fewer, or less fit brood, as SINV-1 infection in *S. invicta* queens has been shown to negatively affect egg formation (Valles and Hashimoto, 2009), but to study this effect may require dissecting the queens, which is not feasible for a 30 day study. While the direct mechanism for worker mortality in SINV-1 infected *S. invicta* colonies may not be clear, the presence of SINV-1 in laboratory colonies of *S. invicta* was significantly increased worker mortality over time, which may lead to an earlier colony collapse when compared to similarly sized, uninfected colonies. Future studies should be conducted to examine the direct effects of SINV-1 infection on fitness and behavior of whole *S. invicta* colonies, and if the use of dsRNA as a tool to reduce viral titers of SINV-1 would reverse these effects.

4.2 Delivery of SINV-dsRNA and Effect on *S. invicta* colony Worker Mortality

SINV-1 positive colonies exposed to SINV-dsRNA on day 15 were shown to have a relative decrease in mortality over a 15 day period. By the end of the 30 day period, treatment group mortality was shown to not differ significantly from control mortality. While this decrease in mortality is correlated with the presence of dsRNA, the decrease in mortality is directly caused by exposure to dsRNA or a natural fluctuation in viral pathogenicity was not assessed. A future

study may be used to answer this question by incorporating a second treatment group that is exposed to SINV-1, but not dsRNA. Another possible method for testing the effect of the down-regulation of SINV-1 on *S. invicta* worker mortality would be to treat infected *S. invicta* colonies in the lab with SINV-dsRNA, and comparing the worker mortality to an untreated SINV-1 infected control group. Future studies should be performed that combine a wider variety of treatment and control groups to help answer questions regarding the interaction of SINV-1 down-regulation and mortality in *S. invicta* colonies.

4.3 *S. invicta* Colony Viral Titers

Viral titers of SINV-1 were shown to increase, then decrease in SINV-1 and SINV-dsRNA treated *S. invicta* colonies over the 30 day period, while SINV-1 viral titers remained relatively constant over the same time period in uninfected control *S. invicta* colonies. Worker mortality in SINV-1 treated *S. invicta* colonies and worker SINV-1 viral titers were directly correlated. Fluctuations of viral titers of SINV-1 may be attributed to several factors. First exposure of SINV-1 specific dsRNA was responsible for the decrease in viral titers of SINV-1 in *S. invicta* workers, who would then transfer the dsRNA constructs to the brood via trophallaxis. Second, the natural viral immune response of *S. invicta* against SINV-1 may lead to the reduction of viral titers of SINV-1. The corresponding decrease in *S. invicta* worker mortality may also be due to several factors. Considering that both workers and brood rely on one another to feed, it should not be surprising that increased mortality in one, may negatively affect the other. Future studies should be performed that determine if worker mortality in SINV-1 infected *S. invicta* colonies is primarily the result of brood loss or negative impacts to worker fitness.

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Chapter Four

Micro-RNA Expression as a result of Solenopsis invicta Virus-1 Infection

1. Introduction

Innate immunity may be the most conserved defense mechanism against pathogens. In vertebrates, the innate immune system provides an initial response to infection, and is a key component in priming the adaptive immune response. Insects lack adaptive immunity and rely solely on innate mechanisms in response to viral infections. RNA interference (RNAi) is an intrinsic cellular defense mechanism that regulates RNA viruses in plants and insects, and is one of several RNA-dependent gene silencing pathways (Ding and Voinnet, 2007). RNA-driven silencing is initiated by an RNase III enzyme Dicer, which cleaves longer precursor double-stranded RNA (dsRNA) sequences into smaller RNA duplexes approximately 21-23 nucleotides in length (Bernstein et al., 2001; Zamore et al., 2000). The small RNA duplexes formed this way are next incorporated into an RNA-induced silencing complex (RISC), in which only one strand is retained (Schwartz et al., 2003). This retained guiding strand is used to direct RISC to a complementary target, which is then degraded by the post-transcriptional gene silencing protein Argonaute (Ago) (Hutvangner & Simard, 2008).

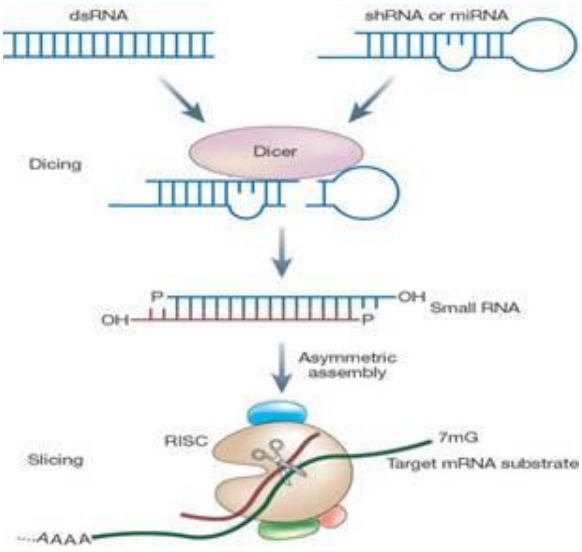

Figure 9**.** Summary of RNAi and miRNA silencing pathway courtesy of Welgen Inc.

Small-interfering RNAs (siRNAs) generated by Dicer-2 (Dcr-2) and incorporated into Ago2-dependent RISC, are ultimately utilized in the degradation and silencing of complementary messenger RNA (mRNA) and viral RNAs. In comparison, microRNAs (miRNA), a class of small (22bp) RNA sequences that participate in the regulation of biological processes, are processed and integrated into similar proteins to those in the RNAi pathway (Bartel, 2004). Endogenous primary miRNAs (pri-miRNA), transcripts formed from the introns of transcribed genes and processed in the nucleus by the enzymes Drosha and Pasha (Denli et al., 2004), are cleaved by Dicer-1 (Dcr-1) into mature miRNA (Lee et al., 2004). Mature miRNA are incorporated into RISC via and Ago-1 mediator, and are utilized in the inhibition of complementary transcripts (Okamura et al., 2004). In insect species of the order hymenoptera, miRNA mediated transcript regulation is highly conserved and regulates processes including behavior, caste differentiation, development, metabolism, reproduction and innate immunity (Varghese & Cohen, 2007; Varghese et al., 2010; Xu et al., 2003).

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera, Formicidae), is an invasive ant species from South America that arrived in the United States between the 1930's and 1940's aboard shipping containers in Mobile, AL (Trinskel, 2006). As of 2001, *S. invicta* populations have spread to approximately 128 million hectares across the southern United States (Williams et al., 2001), with an even greater potential range that is primarily limited by regional temperature (Callcott & Collins, 1996). The range, mound size, density of ants per mound, and population density of *S. invicta* is significantly greater in the United States than that of native South America (Porter et al., 1992; Porter et al., 1997). These differences are likely the result of a lack of natural enemies in the U.S. relative to native South America (Williams et al., 2003; Jouvenaz, 1983), and potential habitat suitability difference (Porter et al., 1997). In 2004, the first of three viruses ever known to infect *S. invica*, the *Solenopsis invicta* virus-1 (SINV-1), was discovered (Valles et al., 2004).

SINV-1 is a single stranded, positive sense, polyadenylated, picorna-like member of the type IV insect infecting viruses *Dicitroviridae* (Bonning, 2009). This combination makes the SINV-1 replication process similar to that of the host mRNA. Little is known of the pathogenicity of SINV-1 to *S. invicta*, which presents as a chronic, asymptomatic infection that may transform into a lethal state after infected *S. invicta* individuals are secondarily stressed by another infection, significant environmental change (Oi & Valles, 2009; Bonning, 2009). SINV-1 is a part of the family of viruses that is associated with honey bee mortality. This provides evidence that SINV-1 may have application as a self-sustaining, long term, biological control agent against *S. invicta*.

In this study the existence of predicted *S. invica* miRNAs was validated and their expression was measured in SINV-1 infected adult *S. invicta* workers exposed to SINV-1viral

isolate and SINV-1 specific dsRNA. The highly conserved nature of miRNAs when compared to the genes that they originate from, allows for relatively strong accuracy when predicting miRNA function (Bartel, 2004). Known miRNAs from the European Honey Bee, *Apis meliferia*, another Hymenopteran, were used as the basis for predicting the presence of the same miRNAs in *S. invicta.* By identifying the existence and potential expression fold changes of miRNAs influenced by viral levels of SINV-1, a link may be found between mechanisms of the *S. invicta* innate immune system and miRNA expression in response to viral infection. To reduce the chance of differences in miRNA expression due to genetic differences, SINV-1 viral titers were manipulated using a combination of RNAi targeting viral capsid genes of SINV-1, and whole isolated SINV-1. The identification of genes and interactions pertaining to SINV-1 infection in *S. invicta* may provide answers to how this virus causes mortality, as well as possible gene knock-down targets for use in combination with SINV-1 as a potential *S. invicta* management tool.

2. Materials and Methods

2.1 Colony collection and maintanance

S. invicta colonies were collected in August, 2013 from Smith County, TX. Between 5,000-10,000 individuals made up of workers, brood and queens were excavated from seven colonies and placed in five gallon containers. The top three to five inches of each container was coated with INSECT-a-SLIP™ (BioQuip Products, Rancho Dominguez, CA) to prevent individuals from escaping during the separation process. Adults and brood were displaced from the soil by slowly dripping tap water into each container, them removing the colony when they floated to surface. Each colony was placed in individual 29- by 15- by 11-cm clear plastic shoe boxes, which were coated with 2 to 3 inches of INSECT-a-SLIP™. A smaller 11- by 11- by 4-

cm clear plastic box with the bottom coated with 3mm Faststone™ (Atlantic Dental Supply, Durham, NC) dental plaster were placed into the larger colony containers to serve as brood chambers. The dental plaster in each brood chamber was saturated with autoclaved tap water prior to colony introduction to maintain humidity. Each colony was maintained in the laboratory (22.5 °C, 12 h L:D), and received a diet modified from Dussutour and Simpson (2008) as needed, with unlimited (autoclaved) tap water.

2.2 SINV-1 Viral RNA Extraction and Screening

To screen for the presence of SINV-1, total *S. invicta* RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacturer's protocol. Five individual workers were used in each extraction and replicated three times for each colony. DNA complementary (cDNA) to a sequence of the SINV-1 genome was produced by a one-step reverse transcriptase PCR (qRT-PCR) (Invitrogen, Carlsbad, CA), then amplified using primers designed by valles and Betine (2011) (F,5′-CATCGAGAT- CTATTGCTACCC-TTCCAAATGCA-3′, R, 5′-ACTTACCTACGCCACTTTCACCAAA- CAACCA-3′). This RT-PCR was performed using the following thermal profile: 50°C for 10min, 95°C for 5min, denatured 95°C for 10 s, annealed 60°C for 30 s, repeated 40 times, followed by a melt curve obtained by ramping from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, then a hold at 4°C. *S. invicta* colony samples that had sequences amplify by RT-PCR and produced a corresponding band length of 148bp on a 2% agarose gel were considered to be virally positive.

2.3 Isolation of whole virus

SINV-1 virions were isolated from SINV-1 infected *S. invicta* colonies using a method modified from Hunter et al. (2003). Approximately 5,000-10,000 infected individual ants were homogenized in 50ml of 0.01M phosphate buffer pH 7.2 containing 10mg of diethyl thio carbamic acid (DETCA) (Sigma-Aldrich, St. Louis, MO), then centrifuged for 20 mins at 300xg. The supernatant was transferred and rotated at 124,500xg for four hours at 4°C. The resulting pellet was dissolved in a solution of 750µl of 0.01 M phosphate buffer containing 0.4% sodium-deoxycholic acid (Sigma-Aldrich, St. Louis, MO) and 4% polyethylene glycol hexadecyl ether (Brijj-52) (Sigma-Aldrich, St. Louis, MO), then centrifuged at 300xg for 15 min. This solution was then dripped through a 0.45 µ filter, at 35°C, and then placed in a dialysis membrane immersed in 4°C nano-pure water for approximately 5-6 hours. The precipitate that formed in the dialysis tubing was collected and stored at -80°C, with approximately 1ml of hole viral extract recovered. The presence of SINV-1 virions was confirmed with NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA), qRT-PCR, and gel electrophoresis.

2.4 Synthesis of dsRNA

Targeted dsRNA specific to SINV-1 viral capsid was synthesized from total RNA extracted using TRIzol reagent as per the manufacture's protocol from SINV-1 infected *S. invicta* individuals. Specific primers (5′-TGGG- TGGCATAACGCTTGAT-3′ and 5′-CCAGTACTTGCATGGTCC-3′) were used in qRT-PCR to produce a cDNA amplicon of 248bp, which was run through a 2.5% agarose gel and the corresponding band was excised using a QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). A second, identical qRT-PCR was used to amplify the 248bp SINV-1 cDNA capsid sequence fragment using specific 5′-T7-

appended PCR primers (5′-TAATAC GAC- TCACTATAGGGTG- GGTGGCATAAC GCTT-GAT-3′ and TAATACGACTCA CTATA- GGGCCAGTACTTGCA- TGGTCC-3′). This T7 amplified fragment served as the template for dsRNA synthesis using the HIScribe T7 *In Vitro* Transcription Kit (New England BioLabs, Ipswich, MA).

2.5 Delivery of Whole Isolated SINV-1 and dsRNA

A single SINV-1 infected *S. invicta* colony (approximately 10,000 individuals) was selected to produce varying levels of viral titers in SINV-1 infected *S. invicta* workers. Five randomly selected workers for this infected colony were separated into a Petri dish with a single piece of Whatman filter paper cut into a 1/8" piece, and then fasted for one day. The filter paper pieces that were placed in each petri dish were saturated with 100 µl of one of three treatments, water, synthesized SINV-dsRNA (50ng/µl), and whole viral extract (50ng/µl). This process was replicated five times for each treatment. After five days, all 75 workers used in this trial were collected, sacrificed, and washed in 95% ethanol and autoclaved Nanopure tap water to ensure no virus was on the outside of the individuals. Total RNA from individuals from each dish was isolated using TRIzol reagent, and viral titers of SINV-1 were determined using the qRT-PCR SINV-1 screening method described above with the inclusion of a standard curve.

2.6 Production of a Standard Curve

The standard curve used in this study was produced by amplifying a sequence of SINV-1 using the SINV-1 screening primers from Valles and Bextine (2001) (described above), using RT-PCR. The resulting cDNA fragment was run through a 2.5% agarose gel and the band excised using a QIAquick Gel Extraction kit. The extracted amplicon was used as a template for an identical PCR protocol to the SINV-1 screen, as well as an identical gel extraction procedure,

and then diluted in 10 sequential 1/100µl serial dilutions. The concentration of each dilution was determined using Nanodrop 1000, which were then used to calculate the number of amplicon copies in each dilution. Five of these known dilutions were incorporated into each q-PCR to serve as known standards used to calculate the number of viral transcript copies in each unknown sample. Pairwise t-test and Tukey's post-hoc tests were used to determine significant differences in the number of viral transcripts in water, SINV-dsRNA, and SINV-1 treated groups.

2.7 Predicting *S. invicta* miRNAs

To predict the existence of miRNAs in *S. invicta*, known pri-miRNAs from *A. melifera* were taken from MirBase.com and blasted to the *S. invicta* genome on NCBI. The scaffold locations for potential *S. invicta* miRNAs were identified by mapping *A. melifera* pri-miRNAs to the *S. invicta* genome using BLASTn with an E-value cutoff of 0.0001. The known pri- and mature *A. melifera* miRNA sequences and the corresponding *S. invicta* scaffold sequences which matched were imported into Genious V8.0 and pairwise aligned. The resulting predicted pri-miRNA sequences from the *S. invicta* genome were imported onto the RNAFold web server to determine is the appropriate mature miRNA sequence was located into the appropriate double-stranded region and not as part of the hairpin loop (Gruber et al., 2008). Mature miRNA sequences that fell within the appropriate pri-miRNA regions were considered likely to be real and were used as templates for primer design.

2.8 Verification of the Existence of Predicted miRNAs

The cDNA used for screening for the presence of predicted *S. invicta* miRNAs was synthesized using an NCode ™ miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Each reverse transcription reaction was performed with 800ng of template RNA extracted

from *S. invicta* workers. Out of the 110 predicted miRNAs, the existence of 90 were tested for using the predicted mature *S. invicta* miRNA sequences as the forward primer in combination with a universal reverse primer provided in the miRNA First-Strand cDNA Synthesis Kit (Lot number). Each q-PCR was performed using the following thermal profile: 50°C for 2min, 95°C for 2min, denatured at 95°C for 15sec, annealed at 60°C for 30sec, a hold at 72°C for 30sec, repeated over 40 cycles, followed by a melt curve obtained by ramping from 50°C to 90°C by increasing the temperature 1°C each step every five seconds, then a hold at 4°C. The housekeeping gene U6 was included in each q-PCR as a tool to normalize the critical threshold (CT) values of unknown samples to. Each miRNA screened was duplicated in PCR and the relative abundance of each was graphed.

2.9 Determining Selected miRNA Expression Differences

To determine if significant differences existed between the expression of miRNAs due to varying SINV-1 viral titers, five miRNAs of note (Sin-mir-1, Sin-mir-316, Sin-mir-8, Sin-mir-277, and Sin-Bantam) were selected because of their known link to innate immunity in other insect species. Each selected miRNA was used screened via q-PRC following the protocol described above, using template cDNA synthesized from water, SINV-dsRNA, and whole SINV-1 isolated treated *S. invicta* RNA. Each miRNA, and the housekeeping gene U6, was screened twice for each of the three treatments and replicated five times. The averaged CT values of each miRNA were normalized to U6 and significant differences in fold expression were noted using pair-wise t-tests.

3. Results

3.1 Delivery of Whole SINV-1 Isolate and SINV-dsRNA

When compared to SINV-1 infected workers that had been exposed to water, SINV-1 infected, SINV-dsRNA treated *S. invicta* workers were shown to have significantly lower viral titers (figure 10). In comparison, SINV-1 infected *S. invicta* individuals exposed to whole SINV-1 virions had significantly higher viral titers of SINV-1 than similarly infected *S. invica* individuals exposed to water ($p<0.05$) (Figure 10).

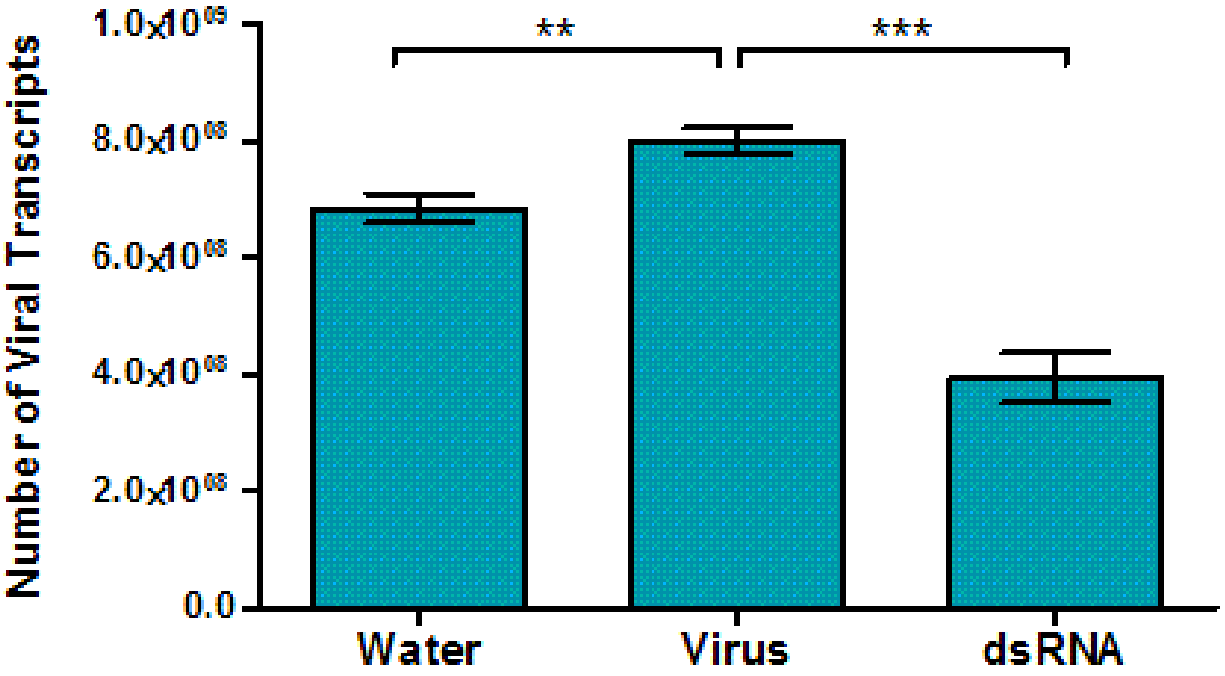

Figure 10**.** Abundance of SINV-1 viral transcripts between water control, dsRNA treated (***, $p<0.0005$), and SINV-1 treated (**, $p<0.005$), SINV-1 positive *S. invicta* workers. Standard error was included on each graph and significance was determined by ANOVA.

3.2 Verification of Predicted *S. invicta* miRNAs

Out of a total of 254 known *A. melifera* miRNAs retrieved from MirBase, 110 aligned to scaffolds in the *S. invicta* genome restricted to an e-value of <0.0001 and were used to design primers to verify using q-PCR (Table 1). Out of the 110 miRNAs predicted, 90 were screened using q-PCR and any sequence that amplified was considered to be a real miRNA in *S. invicta*. Table one below includes a list of all predicted and screened miRNA sequences, and if their existence was verified.

Table 1

Name	Sequence	Existence Verified
Sin-iab-4	ACGUAUACUGAAGGUAUACCGG	yes
Sin-Bantam	UGAGAUCAUUGUGAAAGCUGAUU	yes
Sin-let-7	UGAGGUAGUAGGUUGUAUAGU	yes
Sin-mir-1-2	UGGGAAGUAAGGAAGCACGGAA	yes
Sin-mir-10	ACCCUGUAGAUCCGAAUUUGU	yes
Sin-mir-100	AACCCGUAGAUCCGAACUUGUG	yes
Sin-mir-1000	ACAAUGUCCCAUCACACCAGU	yes
Sin-mir-11	CAUCACAGGCAGAGUUCUAGUU	no
Sin-mir-1175B	CAGAGACCACUUCUCCACUUGA	no
Sin-mir-12	UAAGUAUAACACAAGUACUGGU	no
Sin-mir-124	UAAGGCACGCGGUGAAUGCCAAG	yes
Sin-mir-125	UCCCUGAGACCCUAACUUGUGA	yes
Sin-mir-133	UUGACCCGUGUUCAACCAGCU	yes
Sin-mir-137	UAAUUCCCCAAGAAUACGCGUC	yes
Sin-mir-13a	UUCCACAACCAAUUUGAUGUA	no
Sin-mir-13b	CGCCACAACCAAUUUGACGAUU	yes
Sin-mir-14	UCAGUCUUUUUCUCUCUCCUA	yes
Sin-mir-184	UGGACGGAGAACUGAUAAGGGC	yes
Sin-mir-190	UAAUAUGUUCGAUUCUCGGUCGAUCAUU	no
Sin-mir-193	UACUGGCCUGCUAAGUCCCAA	yes
Sin-mir-2-1	UAUUACAGCCACUUUGUGAGCAGC	no
Sin-mir-2-2	CAUCACAACCACUCUGAUGGGA	yes
Sin-mir-210	UUGUGCGUGUGACAGCGACUA	yes
Sin-mir-219	UGAUGUCCCCACACAAUUCUUG	no
Sin-mir-252a	UAAGUACUAGUGCCGCAGGAG	yes
Sin-mir-252b	CUAAGUAGUAGUGCCGUAGGUGA	yes
Sin-mir-263a	UCCAGUGCCAUUUACAGAAUCCAA	no

Table 1 continued

Sin-mir-263b	GAUGGCAUUGGAUGAUCCAC	no
Sin-mir-275	UCAGGUACCUGAAGUAGCGCGCG	yes
Sin-mir-276	UAGGAACUUCAUACCGUGCUCU	yes
Sin-mir-277	UAAAUGCACUAUCUGGUACGACA	yes
Sin-mir-278	UCGGUGGGACUUUCGUCCGUUU	no
Sin-mir-2788	CAAUGCCCUUGGAAAUCCCAAA	yes
Sin-mir-2796	GGAGGCCGAUAGAAACCCCUUUC	no
Sin-mir-279a	UGCACUAGACCGCCACUCAUU	yes
Sin-mir-279c	ACUAGAGUACACCCGUCCA	no
Sin-mir-279d	GGACUGAAUUCAAACCCAUCCA	no
Sin-mir-281	UGUCGAUGGAUAGCUCUCUUCAU	no
Sin-mir-282	GGUUUAGCCUCUCCUAGGCUUUGUCUGU	no
Sin-mir-283	AAUUACCAGCUGAUAUUU	no
Sin-mir-29b	UAGCACCAUUAGAAAUCAGU	yes
Name	Sequence	Existence Verified
Sin-mir-305	AUUGUACUUCAUCAGGUGCUCUG	yes
Sin-mir-306	UCAGGUACUGAGUGACUCUGAG	yes
Sin-mir-307	UCACACCCAGGUUGAGUGAGUCC	yes
Sin-mir-315	UUUUGAUUGUUGCUCAGAAAGC	yes
Sin-mir-316	UGUCUUUUUCCGCUUUGCUGCCG	yes
Sin-mir-317	UGAACACAGCUGGUGGUAUCUCAGU	yes
Sin-mir-31a	GGCAAGAUGUCGGCAUAGCUGA	no
Sin-mir-33	UUGCACUGCAGGAGUAUUG	no
Sin-mir-34	UGGCAGUGUGGUUAGCUGGUUG	yes
Sin-mir-3477	AAACCCCACGAGGUAAUAUGAG	no
Sin-mir-3715	UUGAUAAACGGAGCAUAAUACUU	no
Sin-mir-3727	UCAGCGGGAGCACGAUCGCGAA	yes
Sin-mir-3728	CCGUCCUUCGCGGCCGCGGUUCG	no
Sin-mir-3736	AGCAUACUCAGGGUAAUCGCC	yes
Sin-mir-3737	GGAAAAAAUUUUUCAUAUACC	no
Sin-mir-375	GUUGAUCGUUGGGAUCGAUGGA	yes
Sin-mir-3753	CGUACAUCCUGAAGCGGCACUU	no
Sin-mir-3756	CCAAUACUUUUUUGUAAGAAUGU	no
Sin-mir-3758	AGCGGCCCACCAGGACCAGGU	yes
Sin-mir-3759	GAGUCCCGCACCAGCAGGUGCCCCC	yes
Sin-mir-3768	CCGAAGACUUUUCCGCACAGCGC	yes
Sin-mir-3770	AAUCCUGCAUCAAGUGCGUUGC	no
Sin-mir-3772	GAUCUAGAUCGAGGGACUUGAA	yes
Sin-mir-3777	UGGAAACAUUUCUUCCUCGGGA	no
Sin-mir-3782	GCUGCAGAGGCACUUGGCCGACAC	yes
Sin-mir-3783	CACUCUCACUUGUUUGGUGGGAU	no
Sin-mir-3785	CGCCGCUAUUACGUCGUGAGGCC	yes
Sin-mir-3786	AUCGUCCUGAGCCAUACAGAGUG	yes

Table 1 continued

Sin-mir-3788	GUUUCGUUAUUCUGCCCUUU	no
Sin-mir-3800	CGAGCCCGCUGAUUGUGCCAUA	yes
Sin-mir-6012	UUCGGCGAUGAGAUCAGCCUGU	not verified
Sin-mir-6037	CAAGCUCUAUGAACUAUUACC	not verified
Sin-mir-6038	UAUGUUUCUGUCUUAUUUCAUU	not verified
Sin-mir-6039	GGUCCAACGCGCGUGUCUACGU	not verified
Sin-mir-6048	AGAGACGAGGUGGACCGACUGGU	not verified
Sin-mir-6049	GGCGGGGGAGGAAUAGACGGU	not verified
Sin-mir-6060	ACCAUGACGAUUACGACAGCGC	not verified
Sin-mir-6065	CUGGAAUGCGAUCCCCCCGGUA	not verified
Sin-mir-6067	CGAAUCCCGCUUUUUGUGA	not verified
Sin-miR-7	GGAUGAUUAGUGAUUCCUUGUUAC	no
Sin-mir-71	UGAAAGACAAGAUAGUGA	no
Sin-mir-750	CUAGAUCCUCACUUCCAACUG	yes
Name	Sequence	Existence Verified
Sin-mir-8	UAAUACUGUCAGGUAAAGAUGUC	yes
Sin-mir-87-1	AAAAGCAAAUUUCAGGCGU	not verified
Sin-mir-87-2	CCGAGCAAAGAGUCAGGCCC	not verified
Sin-mir-927a	UUUCAGAUUUCAAACGCUUUGCC	not verified
Sin-mir-927b	UCUCAAAAUUAUAACGUAACGC	not verified
Sin-mir-928	UUGGUUGUGGAAGUUGGCCAA	not verified
Sin-mir-929	AUUGACUCUAGUAGGGAGUCC	not verified
Sin-mir-92a	AUUGACACAUAUCUCGACCUAU	no
Sin-mir-92b-1	AUUUGCAUUAGUCCAGACCUAA	no
Sin-mir-92b-2	AUUUGCAUUAGUCCAGACCUAA	yes
Sin-mir-92c	AGGCCGGGACAAGUGCAAUUUG	no
Sin-mir-932	UCACUCCCGCGGUGCUUGCAG	yes
Sin-mir-971	UUGGUGUUCUACCUUACAGUGAA	yes
Sin-mir-980	GUGUUGCCCUUCAAAAGGCAGCU	yes
Sin-mir-981	UUCGUCGACAACGAACCAAGUU	no
Sin-mir-9865	UGAUCUAUGUGGUGAUUGUGC	not verified
Sin-mir-9886	CCGGCGACGCCCAGCAAAAGCG	not verified
Sin-mir-9887	AAACGUCGGCGUGGACGGGA	not verified
Sin-mir-9888	UGAUGGUCAAGCAUGGAACGCC	not verified
Sin-mir-9894	AAGGGUUGUGACGGGGGAAA	not verified
Sin-mir-993	AAAGCCCGGAUCUACAGGGUAGAU	yes
Sin-mir-996	UGCACCACAUCCACACUCGUCC	yes
Sin-mir-9b	UCUUUGGUAAUACAGCUCUAUGA	yes
Sin-mir-9c	UAAAGCUAGAUCAGCAAAGGA	yes

Table 1**.** List of predicted mature miRNA sequences in *S. invicta* with notation of existence was verified (yes), unverified (no), or untested (not verified).

3.3 Pri-miRNA Folding and Comparison to the SINV-1 Genome

Out of the 110 predicted *S. invicta* pri-miRNA sequences imported into the MirFold web server, 76 were predicted to fold into the appropriate hairpin shape (data not shown). One miRNA sequence (miR-316) was unique, being the only pri-miRNA sequence predicted to produce a mature sequence complimentary to a sequence of SINV-1 (Figure 11). An example of proper folding compared to improper folding can be found below (Figure 12). After screening for the existence of each predicted *S. invicta* mature miRNA, the relative abundance of each was graphed (Figure 13).

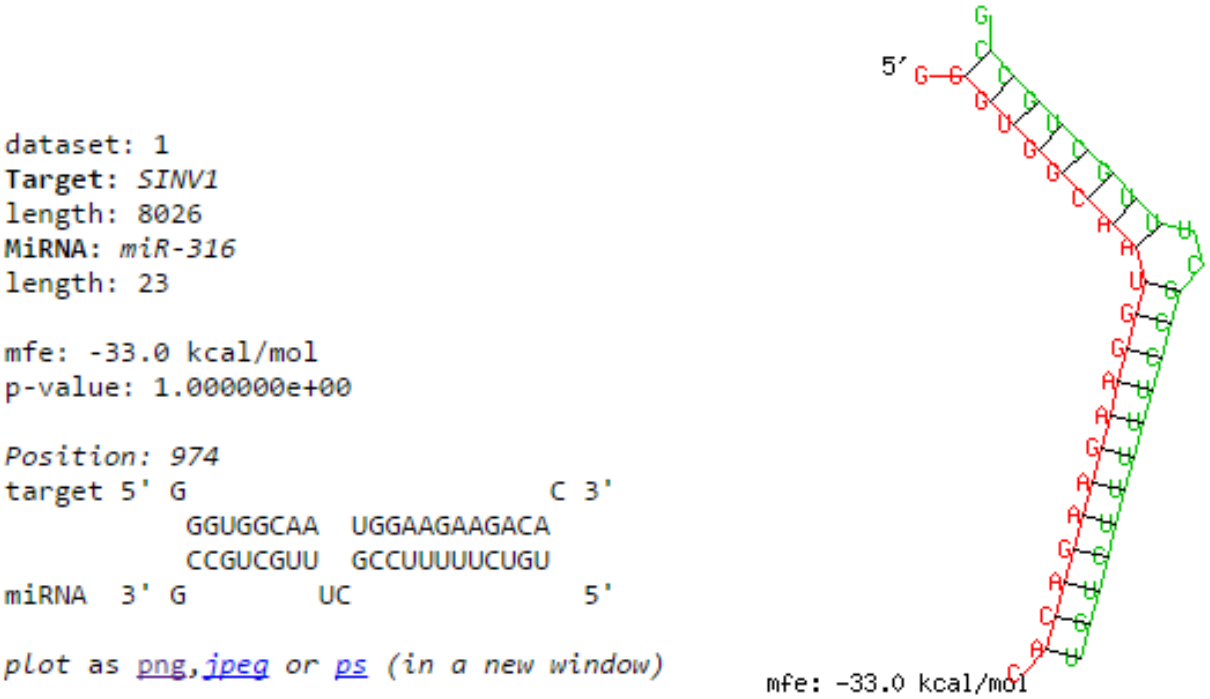

Figure 11. Depiction of the sequence of the mature miRNA miR-316 and its conformation to the SINV-1 genome

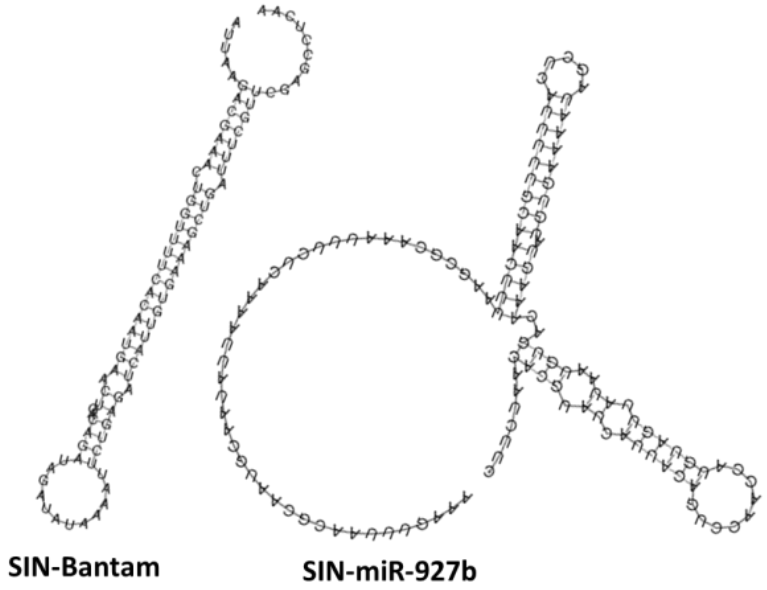

Figure 12. Comparison of a properly folded pri-miRNA sequence (Sin-Bantam), compared to an improperly folded pri-miRNA sequence (Sin-miR-927b).

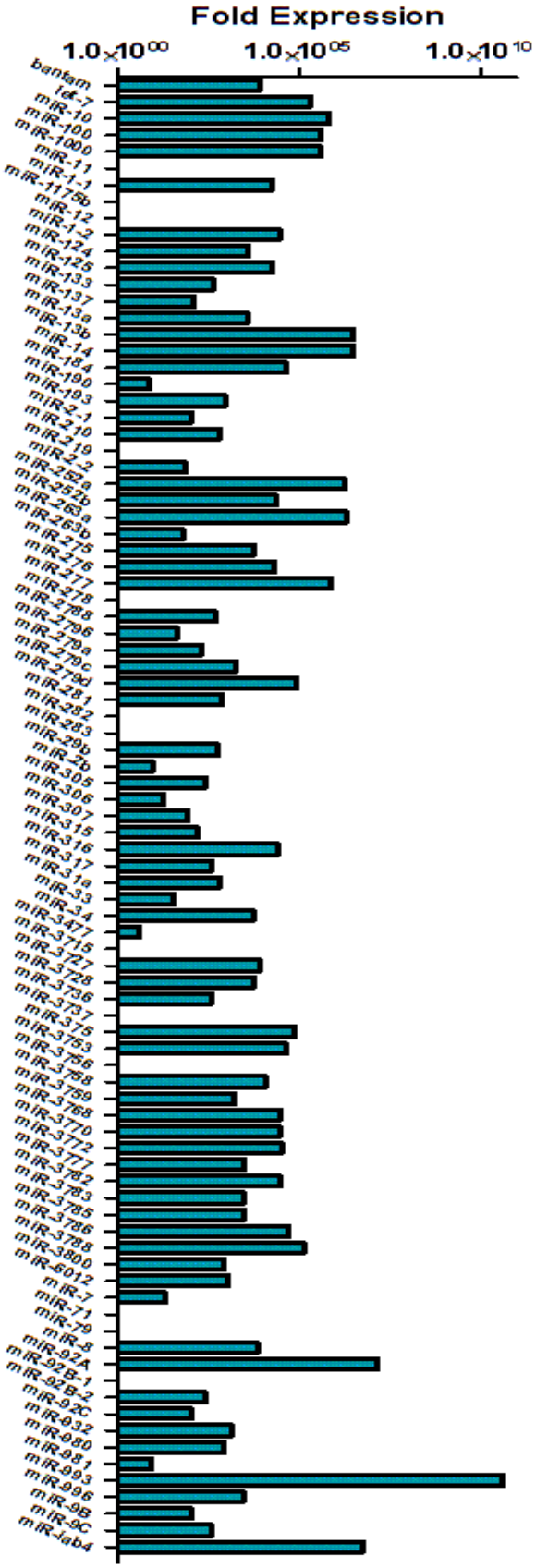

Figure 13. Relative abundance of the 90 screened predicted mature *S. invicta* miRNA sequences using q-PCR

3.4 Secondary Selected miRNA Screening

The five miRNAs selected for further screening were mir-277, mir-316, mir-8, mir-1, and Bantam (Figure 14). While Bantam, mir-277, mir-8, and mir-1 all showed similar trends in that the highest expression was in dsRNA treated workers with the lowest expression found in water treated workers, only Bantam and mir-8 had significant differences in expression fold based on treatment. Both Bantam and mir-8 were shown to have significantly higher expression in dsRNA treated *S. invicta* workers when compared to water treated workers ($p<0.05$). It is also notable that while there was no significant difference between the expression of mir-316 between any treatment group, the lowest expression levels were noted in whole viral isolate treated *S. invicta* workers.

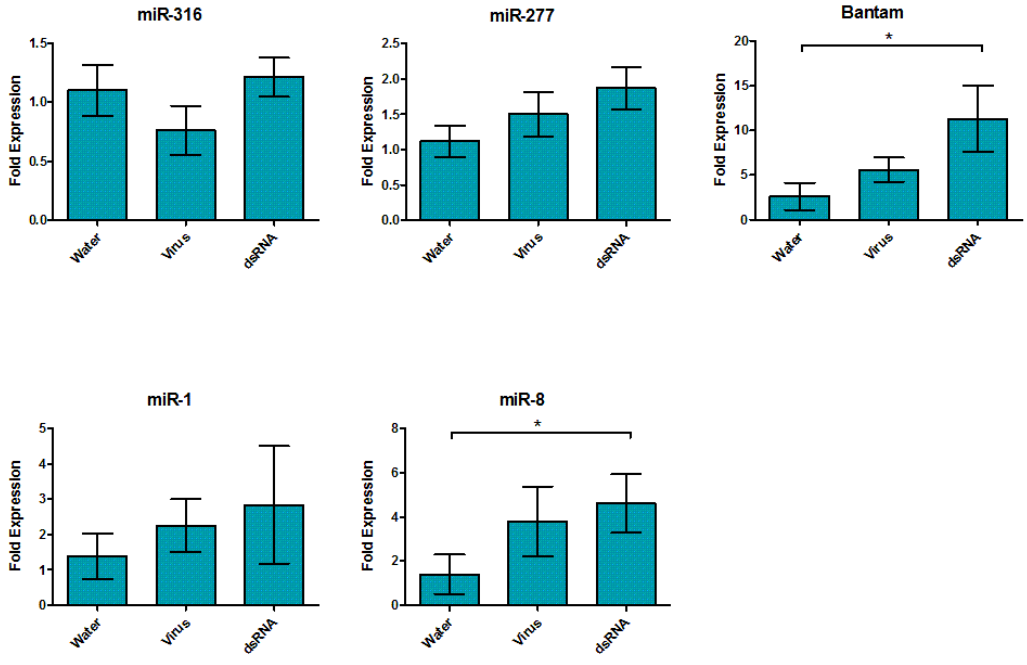

Figure 14**.** Expression differences for each of the five selected mature miRNA sequences across one of three treatments (Water, SINV-1 viral isolate, and SINV-dsRNA). Significance was determined using ANOVA and standard error is represented for each graph.

4. Discussion

4.1 Treatment with SINV-1 Viral Isolate and SINV-dsRNA

By using a combination of SINV-1 specific dsRNA and whole isolated virions viral titers of SINV-1 in infected *S. invicta* workers was increased and decreased respectively. These treated RNA samples were synthesized into cDNA and utilized as the template to test for significant differences between miRNA expression levels in response to varying viral titers. The benefit to using RNAi as a tool to regulate viral titers of SINV-1 instead of finding different *S. invicta* colonies with naturally differing levels of viral infection is that intra-colony genetic differences are less likely to influence gene expression when compared to the gene expression differences between colonies. However, a downside to this method is that there is no way to produce a verifiable viral negative group which to compare miRNA expression. A consideration for a future study would be to test the differences in miRNA expression in choice miRNAs within SINV-1 infected and uninfected *S. invicta* individuals.

4.2 Prediction of *S. invicta* miRNAs and Pri-miRNA Folding

Out of the 110 predicted *S. invicta* pri-miRNAs, only 74 were considered to fold in a manner that you produce the appropriate hairpin shape to produce the correct mature miRNA sequence. The process of verifying proper pri-miRNA folding is subjective, as there is still relatively little known about how dcr-1 processes pri-miRNA sequences into mature miRNAs, as well as what criteria is required for ago-1 to preferentially bind the appropriate mature miRNA sequence. For these reasons predicted *S. invicta* pri-miRNA folding was only taken as a consideration when determining what miRNAs to verify, as there is a relatively high potential for human error based on this data alone. For future studies, a predictable, quantitative method of

determining the proper pri-miRNA fold should be developed and employed, as this may significantly reduce the total number of mature miRNA sequences to verify using q-PCR.

4.3 Measuring Selected miRNA Expression

Within the 90 miRNAs screened, the five that were chosen for further screening were considered to play an important role in gene regulation in regards to innate immunity against viral infection in insects. In *A. melifera*, mir-1 is considered to be a key component in the regulation of cellular proteins (Weaver et al., 2007), and may be ideal to determine if a cellular response may be occurring due to the presence of a viral infection. Bantam, one of the most well studied miRNAs, is known to regulate the expression of other miRNAs in *Drosophila,* as well as processes associated with cell proliferation (Brennecke et al., 2003). Mir-316 is likely important because of complementarity to a sequence in the SINV-1 genome, suggesting regulatory function in regards to SINV-1 infection exists (Figure 11). Mir-277 was selected because of connection with miRNA processing genes such as dcr-1 and ago-1 in *Drosphila* (Forstemann et al., 2007), both of which have related genes involved with innate viral immunity and gene expression. Finally, mir-8 has been implicated in the innate response against viral infections in insects (Chen et al., 2010).

Four of the five selected miRNAs that were compared across the three treatments, Bantam, miR-277, miR-8, and miR-1, all had similar trends in expression across the three treatments. In all four cases, the lowest miRNA expression level was noted in water treated *S. ivicta* workers, with expression levels increasing in whole SINV-1 viral isolate and SINV-dsRNA treated workers respectively. All four of these miRNAs have been implicated in the processing and regulation of cellular machinery, which may help identify why this particular

trend is occurring. It is possible that in SINV-dsRNA treated, SINV-1 infected *S. invicta* workers, miRNAs that are upregulated as a result of the naturally occurring viral infections are also upregulated by the presence of dsRNA. The RNAi pathway, a component of innate immunity in insects, is activated by the presence of dsRNA, a molecule that may naturally form in viral infection as a result of a single stranded RNA (ssRNA) virus forming a hairpin-like shape. Depending on the virus, this hairpin shape may occur in varying frequencies. With the introduction of isolated dsRNA into the cell, it is likely that the machinery that processes dsRNA, viruses, and pri-miRNAs (Dicer, and Ago), are upregulated, leading to an increased expression of miRNAs that regulate these processes. MiR-8, miR-277, miR-1, and Bantam may all be related to the regulation of innate immunity, miRNA regulation, and protein expression in the presence of a viral infection in *S. invicta*.

By having a clear understanding of how miRNAs are expressed in *S. invicta* during a viral infection, it may be possible to locate possible knock-down gene targets that could lead to an increase in viral replication. Among the miRNAs screened and tested, miR-8, Bantam, and were shown to be significantly upregulated as a result of exposure to SINV-1 and SINV-1 specific dsRNA, which could imply the importance of these miRNAs in the *S. invicta* immune system. While not significantly regulated as a result of treatment with SINV-dsRNA or SINV-1 viral isolate, miR-316 has potential to be a significant factor in the innate immune response of *S. invicta* against SINV-1 infection.

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Chapter Five

Conclusions and Future Research

Conclusion

In the United States, invasive species represent one of the largest biological sources of ecological and economic damage. Invasive species often displace, and out-compete native species for food and space, which can significantly reduce biodiversity and alter food webs. Attempts to midigate, control, and repair damage caused by invasive species can cost tax payers billions of dollars (U.S.) each year. *Solenopsis invicta* Buren, or the red imported fire ant, is often considered a classic example of an invasive species as it been implicated in the displacement of native species, and is attributed with approximately six billion dollars (U.S.) in damage, each year in the United States alone.

To date, the attempts of pest management agencies to achieve long-term control of *S. invicta* populations in the United States have failed. The use of chemical control agents against *S. invicta* have only produced a short term, isolated population control, and have been shown to have off target effects on other arthropods. Climate models have been used to predict that the current expansion of suitable habitat for *S. invicta* is increasing, implicating that the ecological and economic damage caused by *S. invicta* will also continue to grow. This is why there is an increasing need to discover a long-term, self-sustained, and targeted biological control agent against *S. invicta* in the United States.

If controlled, the *Solenopsis invicta* virus represents significant potential as a management tool against *S. invicta*, but the use of premeditated and widespread use of a virus for any purpose could be considerably controversial. This is why the preceding research is important, as it answers these concerns. RNA interference is a powerful technology already in

use regulating biological systems in insects and plants. By demonstrating that RNAi could be used as a tool to regulate viral titers of SINV in *S. invicta*, pest management professionals would be provided the justification to utilize SINV as a tool to control populations of *S. invicta.*

The second chapter of this document includes methods that describe how RNAi can be used to down-regulate viral titers of SINV in individual *S. invicta* workers. The third chapter describes methods that expand on this use of RNAi in individual *S. invicta* workers, and applies it to whole *S. invicta* colonies. Not only were viral titers of SINV first increased, then decreased in whole *S. invicta* colonies, but a corresponding increase and decrease in mortality was observed. This is exactly the kind of information that professionals need to make informed pest management decisions. The final chapter of this thesis was used to expand on the effects of SINV infection inside of the *S. invicta* host by examining how gene expression is altered through the exposure to SINV virions, and SINV targeted double stranded RNA. A total of five micro RNAs were identified as significant to the *S. invicta* innate immune system, and two of these were shown to possibly play a significant role in the *S. invicta* immune response against SINV.

This research represents a wealth of possibilities related to the management of *S. invicta* with the use of RNAi, and it is my goal to expand upon the work that I have already completed. By identifying possible miRNAs implicated in the innate defense against SINV, it may be possible to target the corresponding genes that the miRNAs originate from, and perform a knock-down study. If these genes really are linked to SINV infection in *S. invicta*, the act of reducing expression of these genes my increase SINV replication, possibly leading to increased host mortality. One of the problems faced by researchers who study *S. invicta* is the lack of annotations to its genome. More work should be done to examine this genome, and to identify possibly significant gene targets related to the *S. invicta* immune system.