PROCEDURES TO MITIGATE THE IMPACT OF SOLENOPSIS INVICTA VIRUS 3 IN FIRE ANT (HYMENOPTERA: FORMICIDAE) REARING FACILITIES

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Cost estimates for controlling and repairing damage caused by the red imported fire ant, *Solenopsis invicta*, exceed $5 billion annually in the United States (Pereira 2003) and although insecticides are highly effective against this ant pest, they must be used on a regular interval to maintain control. Without consistent insecticide use, fire ant populations invariably re-infest untreated areas. This dependency on insecticide use and the widespread distribution of *S. invicta* throughout the southern tier of the United States illustrate the importance of biological agents in achieving sustainable control of fire ants (Williams et al. 2003).

Viruses and other pathogens are common challenges for insect rearing facilities (Fuxa et al. 1999; Sikorowski & Lawrence 1994). Viruses are also recognized as important components of insect biological control programs (Lacey et al. 2001). However, despite intensive searches by traditional means, no viruses had been identified in *S. invicta* until a metagenomics approach was employed (Valles et al. 2008). With this method, 3 unique positive sense, single-stranded RNA viruses were discovered from environmental samples of *S. invicta*. These viruses include a dicistrovirus, *Solenopsis invicta* virus 1 (SINV-1) (Valles et al. 2004), and 2, as yet, unclassified viruses, SINV-2 (Valles et al. 2007) and SINV-3 (Valles et al. 2010; Valles & Hashimoto 2009). SINV-3 is quite virulent (Chen et al. 2012) and exhibits host specificity for fire ants in the s*aevissima* complex (Porter et al. in review)—characteristics that are ideal for development as microbial agents to control invasive fire ants. An additional advantage exhibited by SINV-3 appears to be environmental persistence. Unfortunately, this characteristic was learned from difficulties rearing fire ants in a laboratory environment contaminated with SINV-3. During the initial characterization of SINV-3 (Valles & Hashimoto 2009), virus-infected colonies were retrieved from the field and maintained in a laboratory rearing facility at the USDA-ARS Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, Florida. During this time, all uninfected *S. invicta* colonies housed in the facility contracted the SINV-3 infection. Attempts to maintain areas free of SINV-3 were unsuccessful and laboratory insectaries repeatedly experienced colony collapses caused by SINV-3 over the ensuing 3 years. We suspected that infectious SINV-3 particles were being disseminated in pieces of dried, friable ant corpses. Unlike SINV-1 and -2, which are limited to the midgut, the SINV-3 infection is systemic (Valles 2012). The extent of this suspected contamination was verified by conducting swab tests of the insectary for the presence of SINV-3 by RT-PCR. Surfaces of the floors and table tops in all 3 rearing areas, each in separate buildings at CMAVE were found contaminated with SINV-3 (mean 83%, n = 15). Indeed, dead ants in colonies often contained greater than 1×10^9 SINV-3 particles per insect. Thus, we set out to document the fire ant rearing difficulties experienced at the USDA insectaries, methods to render the virus inactive, and utilization of these methods to limit the impact of SINV-3 on colonies in fire ant rearing facilities.

Formal studies were conducted to evaluate the effectiveness of different treatments to eliminate SINV-3. *Solenopsis invicta* workers (100 mg) previously determined to be infected with SINV-3 were homogenized in 5 mL of diethylpyrocarbonate (DEPC)-treated water with a mortar and pestle. The slurry was filtered through fine glass wool. Aliquots (100 μL) of the filtrate were transferred to 1.5 ml microcentrifuge tubes. Different treatment solutions or conditions were applied to each aliquot. Specifically, 100 μL of a dilute HCl (pH 1.5), NaOH (pH 12.5), bleach (3.75% sodium hypochlorite), or DEPC-treated water (control) were added and allowed to incubate at room temperature for 1 h. Additional treatments included 1) heating to 100 °C in a block heater for 1 h, 2) autoclaving for 30 min and 3) UV radiation exposure (4,000 μW/cm² [5 joules]) administered with a UV Stratalinker 2400 (Stratagene, Cedar Creek, TX); 100 μL of DEPC-treated water was added to each of these treatments before exposure for equivalency. Each treatment was replicated 3 times using 3 different SINV-3-infected colonies. In addition, each experiment was completed with duplicate determinations for each treatment. The data were analyzed by Dunnett's multiple comparison procedure in which each treatment mean was compared for statistical significance to the untreated control. Immediately after treatment, RNA was extracted from each treatment by the Trizol method (Invitrogen, Carlsbad, California)

following the manufacturer's protocol. The RNA was diluted to 10 ng/µL and then used as template to synthesize cDNA and quantify by real time PCR with a SINV-3 gene specific primers as described previously (Valles & Hashimoto 2009). The data for each replicate were normalized to each respective control (no treatment) and then combined. Data were presented as a proportion of the control quantity. Mean (±SE) number of copies of the SINV-3 genome detected in the control samples was 4.95×10^5 ($\pm 2.01 \times 10^5$) copies of SINV-3 genome/ng RNA.

All treatments significantly reduced the amount of SINV-3 capable of being detected by RT-PCR (Fig. 1). Bleach was the most effective – nearly completely eliminating detection of SINV-3 RNA after a one h exposure. UV light exposure and autoclave treatments reduced SINV-3 detection to less than 2% of the control. However, our objective was to identify treatments that would eliminate SINV-3 so that our rearing facilities could be protected/cleared from these viral infections. Thus, UV treatment and bleach were incorporated in our rearing process and subsequently evaluated.

An insectary to rear fire ants (1.8 × 3.7 m) previously used to study the host specificity of SINV-3 was heavily contaminated by infectious SINV-3 particles as determined by RT-PCR evaluation. All rearing trays and supplies were either discarded or submerged in bleach for 30 min or more with a 0.2% hypochlorite solution. The room was then sprayed and swabbed with a bleach solution (0.5-2%). Tray racks were removed, sprayed with bleach, and allowed to dry from exposure to the sun for several days before being returned to the insectary.

Several dozen small uninfected colonies reared from newly mated queens were transferred into the insectary in Jul 2011. At this point, we implemented rigorous hygiene procedures designed

to block or limit the spread of SINV-3 among *S. invicta* colonies. Three procedures were designed to limit the spread of infectious dust particles: 1) lids were put on all nest trays, 2) an air purifier with a UV sterilizer was installed (Newport 9000, 3 m³/min., UV spectrum ≥ 20µ w/cm² at 10 cm), 3) tray cleaning activities were conducted outside so the dust from cleaning operations would not contaminate the rearing room or nearby work rooms. When cleaning became necessary, 4) we discarded disposable rearing materials such as feeding cups, nest cells, and water tubes and 5) we sterilized reusable materials such as nest trays, lids, and forceps in a bleach solution as described above.

Next, we implemented procedures designed 6) to eliminate mechanical movement of infected particles and occasionally even live workers among colonies during feeding and other maintenance activities. Specifically, food and water could be dropped or squirted into the rearing trays, but items in the trays could not be touched without changing gloves or washing hands prior to moving to the next colony. Occasionally, we stretched this rule if we could immediately remove an item without danger of touching anything else or transporting ants out of the tray. Practically, this meant that dead ants and feeding refuse was allowed to accumulate in the colony trays much more than had been our previous practice.

The final 2 protocols were designed to eliminate infected colonies from the insectary. Specifically, 7) colonies were discarded as soon as brood production declined and they began to appear unhealthy rather than retaining them and trying to "nurse them back to health." Finally, 8) field collected colonies were not permitted in the clean rearing room because of the probability that they might be infected (Valles et al. 2010; Valles et al. 2009). All new colonies were obtained by collecting and rearing newly mated queens on the assumption that SINV-3-infected queens would be unlikely to successfully found new colonies and, as a result, would likely die and be discarded while still in the claustral founding stage.

The result of the hygiene procedures above was that we were able to maintain and conduct tests on dozens of fire ant colonies for over a yr without any of the colonies becoming infected with SINV-3. Several colonies did stop producing brood, but none of these colonies tested positive for SINV-3. Furthermore, none of the colonies in the insectary tested positive for SINV-3 by the end of Aug 2012, 14 months after the hygiene protocols were implemented. In contrast, an insectary in another building at CMAVE where these protocols were not implemented continued to have die-offs as a result of SINV-3 infection.

SINV-3 contamination of our insectaries have made rearing healthy, quality fire ant colonies problematic since the discovery of this virus (Valles & Hashimoto 2009). Obviously, this has
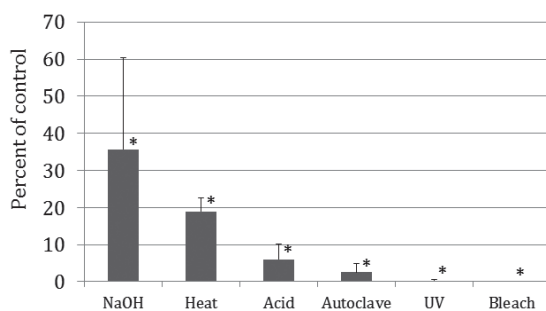

Fig. 1. Effect of different treatments ($n = 3$) on quantities of SINV-3 detected by qPCR. Values are presented as a percentage of the control value (4.95×10^5 SINV-3 genome copies/ng RNA ($\pm 2.01 \times 10^5$). Bars with an asterisk are significantly different ($P < 0.05$) from the control by Dunnett's multiple comparison procedure.

serious implications for a research institution charged with studying fire ant biology and control. Thus, it was imperative that we identify methods to eliminate SINV-3 contamination and implement their use. Common household bleach has been identified as an effective agent for inactivating a number of viruses (e.g., (Greatorex et al. 2010). Our laboratory tests and insectary-wide evaluations also demonstrate the effectiveness of bleach as an important component to maintain areas free of SINV-3. While the methods outlined above increase the required diligence necessary to rear fire ants, they do not substantially increase the labor required for rearing.

Summary

During the initial characterization of Solenopsis invicta virus 3 (SINV-3), virus-infected fire ant colonies were retrieved from the field and maintained in the laboratory rearing facility at the USDA-ARS CMAVE, Gainesville, FL. During this time, all uninfected *S. invicta* colonies housed in the facility contracted SINV-3 infections. Virus infection in fire ant rearing facilities and methods to render the virus inactive and to limit its impact on fire ant rearing are described.

Key Words: SINV-3, bleach, sodium hypochlorite, UV radiation, mechanical movement, infectious dust particles

Resumen

Durante la caracterización inicial del virus 3 de *Solenopsis invicta* (SINV-3), colonias de hormigas de fuego infectadas por el virus fueron recuperadas en el campo y mantenidas en el centro de cría en el laboratorio del CMAVE USDA-ARS en Gainesville, Florida. Durante este tiempo, todas colonias no infectadas de *S. invicta* en la instalación contrataron infecciones de SINV-3. Se describen la infección de cría de hormigas de fuego por el virus en las instalaciones de cria y los métodos para hacer que el virus sea inactivo y limitar su impacto sobre la cria de la hormiga de fuego.

Palabras clave: SINV-3, cloro, hipoclorito de sodio, radiación UV, movimiento mecánico, partículas infecciosas de polvo

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