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Solenopsis invicta virus 3: infection tests with adult honey bees (Hymenoptera: Apidae)

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Abstract

Solenopsis invicta virus 3 (SINV-3) is a positive sense, single-stranded RNA virus that has considerable potential as a self-sustaining or classical biocontrol agent against the invasive fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae) because it can cause substantial mortality in colonies of this species. Based on extensive host specificity tests with other ants, we predicted that SINV-3 would not infect the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). Nevertheless, because of the economic importance of honey bees, it was necessary to confirm this prediction before proceeding with field release of SINV-3 into regions where it does not yet occur. To test our prediction, we 1) examined field bees for the presence of the virus, 2) conducted specific searches of the GenBank databases for sequences (amino acid and nucleotide) with identity to SINV-3, and 3) inoculated laboratory groups of honey bees with large doses of SINV-3. SINV-3 was not detected in field bees or in GenBank libraries associated with honey bees, Apoidea, or non-host Insecta generally. Western blot analysis for SINV-3 capsid proteins showed that viral proteins were not produced in inoculated honey bees, but were produced in inoculated fire ants. Furthermore, qPCR analysis revealed no significant increase in SINV-3 quantity in honey bees, beyond the inoculating dose, whereas virus quantity dramatically increased over time in inoculated fire ants. Based on these results, we conclude that field release of SINV-3 as a biocontrol agent against red imported fire ants in regions without the virus would pose little or no threat to honey bees.

Key Words: *Solenopsis invicta*; host range; host specificity; SINV-3

Resumen

El virus Solenopsis invicta 3 (SINV-3) es un virus de ARN de cadena sencilla, de sentido positivo que tiene un gran potencial como un agente de control biológico autosostenible o clásico contra la hormiga brava invasora, *Solenopsis invicta* Buren (Hymenoptera: Formicidae); porque puede causar una mortalidad substancial en las colonias de esta especie. Basados en pruebas extensivas de especificidad con otras hormigas, predijimos que el virus SINV-3 no podría infectar la abeja melífera, *Apis mellifera* L. (Hymenoptera: Apidae). Sin embargo, debido a la importancia económica de las abejas melíferas, era necesario confirmar esta predicción antes de proceder con la liberación en el campo del virus SINV-3 en regiones donde éste todavía no ocurre. Para probar nuestra predicción, examinamos 1) abejas melíferas colectadas en el campo para detectar la presencia del virus, 2) realizamos búsquedas específicas de secuencias (amino ácidos y nucleótidos) con la identidad de SINV-3 en la base de datos de GenBank, e 3) inoculamos en el laboratorio grupos de abejas melíferas con dosis altas del virus SINV-3. SINV-3 no fue detectado en las abejas de campo o en las bibliotecas del GenBank asociadas con las abejas melíferas, Apoidea, u otros hospederos en la clase Insecta generalmente. Análisis del Western blot para detectar proteínas de la cápsula del virus SINV-3 demostró que la formación de proteínas del virus no ocurrió en las abejas melíferas inoculadas, pero sí en las hormigas bravas inoculadas. Además, el análisis de qPCR no reveló ningún aumento significativo del virus SINV-3 en las abejas melíferas, más allá de la dosis de inoculación; mientras que, la cantidad del virus aumentó drásticamente con el tiempo en las hormigas bravas inoculadas. Basados en estos resultados, concluímos que la liberación de campo del virus SINV-3 como un agente de control biológico contra las hormigas bravas importadas en regiones sin el virus representaría poca o ninguna amenaza contra las abejas melíferas.

Palabras Claves: *Solenopsis invicta*; rango de hospederos; especificidad hospedero-patógeno; SINV-3

Solenopsis invicta virus 3 (SINV-3) is one of 3 RNA viruses discovered from the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (Valles & Hashimoto 2009). It is a positive-sense, single-stranded virus with similarities to Nylanderia fulva virus 1 and Kelp fly virus in the newly proposed family Solinviviridae (Valles et al. 2014a, 2016). Members of Solinviviridae are all insect viruses infecting hosts in the orders Diptera, Coleoptera, Hymenoptera, Phthiraptera, and Psocoptera. The 3 hymenopteran hosts are all ants (*Monomorium*, *Nylanderia*, *Solenopsis*). SINV-3 is stage specific to fire ant workers and does not appear to replicate in larvae or pupae (Valles et al. 2014b), and larvae are not necessary for fire ant workers to become infected (unpublished data).

This virus has considerable potential for use as a self-sustaining biocontrol agent against invasive *S. invicta* populations in the Caribbean, Australia, Taiwan, Mainland China, and other regions where this virus does not yet occur (Valles et al. 2015; Yang et al. 2010). SINV-3 is highly infectious and capable of killing *S. invicta* colonies in the laboratory (Porter et al. 2013; Valles & Porter 2013, 2015; Valles et al. 2013, 2014b). In the field, it has been reported to occur in about 11% of colonies near Gainesville, Florida (Valles et al. 2010) and 10% in Argentina (Valles et al. 2009). SINV-3 infects only South American fire ants in the *S. saevissima* group (e.g., *S. invicta, S. richteri* Forel; Porter et al. 2013). Native North American *Solenopsis* fire ants (e.g., *S. geminata* [F.]*, S. aurea* Wheeler*, S. xyloni* McCook) and *Solenopsis* thief ants are not

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infected even though they are in the same genus (Porter et al. 2015b). Likewise, 16 additional species of ants from 4 subfamilies and 13 genera are not infected by SINV-3, even when challenged with massive doses (Porter et al. 2013; Porter et al. 2015b).

The objective of this study was to determine if SINV-3 is capable of infecting the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). The high host specificity of SINV-3 to fire ants in the South American *S. saevissima* group (see above) suggests that infection of honey bees is extremely unlikely. Nevertheless, the economic importance of honey bees justifies the extra effort to verify this supposition. Honey bees serve as host to a number of serious viral pathogens (Allen & Ball 1996; Stankus 2008). Indeed, honey bees are known to harbor and succumb to numerous positive-strand RNA viruses (Chen & Siede 2007). Furthermore, Argentine ants (*Linepithema humile* [Mayr]) were recently reported to be a common host for Deformed wing virus, a pathogen associated with honey bee mortality (Sebastien et al. 2015). Therefore, in an abundance of caution, we exposed honey bees to extremely large doses of SINV-3 to determine whether they were capable of serving as host to this virus. We also searched GenBank for sequences similar to SINV-3 and tested field-collected bees for the presence of SINV-3.

Materials and Methods

FIELD-COLLECTED BEES

The first test was to determine whether SINV-3 might already occur in field honey bee colonies in the Gainesville area because this virus is relatively common in co-occurring red imported fire ant populations (5–50% of colonies depending on location and season, Valles et al. 2010). Two bees were collected from the entrance of each of 20 colonies at the University of Florida (UF) Bee Biology Research Unit (29.6270°N, 82.3563°W; 21 Aug 2015) and 17 colonies at the Gainesville United States Department of Agriculture (USDA) apiary (29.6354°N, 82.3606°W; 26 Aug 2015). These honey bee colonies were within the foraging territories of surrounding fire ant colonies and potentially exposed to fire ants from hundreds of additional colonies while collecting nectar and pollen. SINV-3 infects fire ant colonies during all months of the year, although rates are typically lowest in the summer (Valles et al. 2010). Total RNA was extracted from individual bees by the Trizol method (Invitrogen, Carlsbad, California) according to the manufacturer's directions. cDNA was synthesized with Superscript III (Invitrogen) and polymerase chain reaction (PCR) was subsequently conducted to amplify a portion of the SINV-3 genome as reported previously (Valles et al. 2009).

GENBANK DATABASE SEARCHES

BLAST analyses were conducted with the SINV-3 genome and fused polyprotein sequence (GenBank accession NC_012531.1) in an effort to identify the SINV-3 sequence (or portions thereof) in the Apoidea and Insecta generally (Altschul et al. 1997). These analyses were conducted in Apr 2016. We hypothesized that transcriptome shotgun assemblies (TSA), expressed sequence tags, nucleotide collections, and other databases in GenBank offered an additional method to screen for possible SINV-3 infections in honey bees, other Apoidea, and Insecta. Thus, various BLAST analyses were completed with these databases. All sequences identified with an expectation score of less than 1 were then further examined by BLAST analysis using the organism taxonomic identification "ssRNA viruses" to determine whether they were related to SINV-3 and related viruses in Solinviviridae (Valles et al. 2016) or, instead, more closely related to other picorna-like viruses such as iflaviruses or dicistroviruses.

LABORATORY INFECTION TEST

We used 4 experimental treatments to determine if SINV-3 can infect honey bee workers: 1) SINV-3 inoculated bees fed a homogenate of SINV-3-infected ants, 2) negative control bees fed a homogenate of uninfected ants, 3) standard bees receiving sugar water, and 4) uninfected fire ant colonies (positive controls) fed the same homogenate of SINV-3-infected ants used for the inoculated bees above. Adult bees were tested because SINV-3 only replicates in adult fire ants (Valles et al. 2014b) and broodless groups of fire ant workers are easily infected by this virus (unpublished data).

We collected 100 to 150 adult bees from each of 6 colonies in the apiary of the UF Bee Biology Research Unit (9:30 a.m., 21 Aug 2015). These bees were primarily nurse bees because they were collected by shaking the bees from 1 to 2 frames containing mostly unsealed brood (Evans et al. 2012; Williams et al. 2013) into cardboard boxes designed for shipping queen honey bees (Part RW-150, Mann Lake Ltd, Bakersfield, California). Three groups of bees were set up from each colony by taking bees as they emerged from a hole in the box and alternately placing them into 1 of 3 hoarding cages until each cage contained about 30 bees (±1.5 SD, ~3.7 g total). The 3 test cages from each colony were then randomly assigned to each of the 3 bee experimental treatments described above.

Hoarding cages were constructed by inverting clear 10 ounce (~300 mL) cups onto the lid of a 10 cm Petri dish and holding them together with a rubber band. These cages were similar to those described by Fleming et al. (2015), which were based on descriptions by Williams et al. (2013), except that each cage was vented by 16 small holes (each about 2 mm) melted in the sides with a heated fork, and a piece of plastic cross-stitch mesh (3.5 × 9.5 cm) was inserted to provide a surface on which the bees could climb. The bees were provided water and sugar water (1.5 M sucrose solution, 44% sugar by weight) using 2 mL centrifuge tubes each with 2 small holes melted in the sides at the bottom by using a heated sewing pin. Two of these tubes (1 for water and 1 for sugar water) were hung in the top of each hoarding cage through holes so that the bees could access the liquid as needed (Kirrane et al. 2012; Williams et al. 2013).

Test cages with honey bees were maintained at 27.9 ± 0.6 °C and 58 ± 5% RH in a darkened chamber. This temperature was chosen to maximize bee survival and because it is known to be an optimal temperature for development of the virus in fire ants (Valles & Porter, unpublished data). All test honey bee groups were starved for 3.5 h before inoculation with the virus on 22 Aug 2015. Dead bees were removed from cages daily. Eleven bees that died before being inoculated and 14 bees that lost their stingers during setup and later died were not included in the mortality analysis.

To verify that the bees were receiving infectious virus, we selected 6 small *S. invicta* colonies to serve as positive controls. These colonies contained 2.5 to 5.0 g of workers and brood (mean = 3.9 ± 1.1 g). We tested pooled groups of 15 fire ant workers from each colony on 21 Aug 2015 by reverse transcriptase PCR (RT-PCR) for the presence of SINV-3 (Valles et al. 2013) and all 6 colonies were confirmed free of the virus a day before being exposed to virus. Our fire ant stock colonies remained consistently free of the virus because of careful rearing hygiene (Valles & Porter 2013). Ant colonies were held in small black boxes with 2 nest tubes (Porter et al. 2015a) at the same temperature as the honey bees, but with a 12:12 h L:D photoperiod. Fire ants were starved for 31 h before treatment. The bees were not starved as long as the ants because they have a much higher metabolic rate and would not have tolerated the 1 to 3 d starvation period we normally use when dosing ants. After treatment, ant colonies received domestic crickets (*Acheta domesticus* [L.]; Orthoptera: Gryllidae) 3 times per week and continuous access to small tubes of 1.5 M sugar water (Porter et al. 2015a).

Honey bees and fire ants were inoculated with SINV-3 by feeding them 1.5 M sugar water adulterated with homogenates of SINV-3-infected fire ant workers. This inoculum was formulated by collecting 0.6 g of live workers from each of 3 infected fire ant colonies. These workers were combined and homogenized with a mortar and pestle and then mixed with 1.5 M sugar water (sucrose) at a 1:15 ratio (ants to sugar water) by weight. This mixture was then squeezed through synthetic organza fabric with openings of 0.18 mm to remove large ant body parts. Four aliquots of the inoculum were saved to test for SINV-3 concentration with quantitative PCR (qPCR). Pilot tests with food coloring (McCormick Inc., Maryland) confirmed that our sugar water inoculum with homogenated ants was readily ingested by the honey bee workers and shared with nest mates. The 6 inoculated honey bee groups (1 from each colony) and the 6 fire ant colonies (positive controls) were exposed to freshly prepared inoculum at 2:00 p.m. on 22 Aug 2015. An equivalent sugar water preparation with uninfected ants was presented to the 6 negative control groups of honey bees. The 6 standard honey bee groups received only 1.5 M sugar water. After each group of bees had consumed at least 1 mL of their initial liquid sugar preparations, these feeding tubes were replaced with fresh tubes of plain sugar water. Thereafter, sugar water tubes and water tubes were replaced as needed. A 1 mL cup with a 1:1:1 mixture (by weight) of retail honey, dry pollen (USDA apiary), and Standard 15% Pollen Patty (Global Patties, Butte, Montana) was also placed in the bottom of each cage after inoculum doses were removed.

To test for SINV-3 infections, we collected 3 bees from each of the 6 inoculated groups on days 2, 5, and 9 (9 total bees from each group). Equal numbers of bees were also removed from the negative control groups and the standard groups so that the number of bees in these groups would remain comparable to the inoculated groups. Two groups of 15 small fire ant workers were collected from each of the 6 fire ant colonies on the same schedule as above. RT-PCR and qPCR for the detection and quantitation of SINV-3 were conducted as described previously (Valles & Hashimoto 2009; Valles et al. 2009). Production of newly formed SINV-3 capsid proteins was confirmed by Western blot analysis (Valles et al. 2014b). qPCR and Western blot analysis were conducted by cutting the honey bee in half sagittally; one half was used for RT-qPCR analysis and the other half was used for Western blotting. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 4 to 20% gradient gel and then electroblotted onto a polyvinylidene fluoride membrane. Blots were probed with a polyclonal antibody preparation that recognizes a SINV-3 capsid protein (Valles et al. 2014a).

Mean number of SINV-3 genome copies in SINV-3 inoculated bees at 3 sample days (Fig. 1B) was compared with a 2-way ANOVA in a randomized block design where source colony was the random factor (colonies A–F) and day (2, 5, 9) was the fixed factor. Mortality of test bees was similarly analyzed with a randomized block design with source colony as the random factor (colonies A–F) and treatment (inoculated, negative control, standard) as the fixed factor.

Results

FIELD BEES

Despite collecting from areas where SINV-3 is detected regularly among *S. invicta* ants (Valles et al. 2010), none of the honey bee samples collected from the UF apiary (n = 40 bees from 20 colonies) or the USDA apiary (n = 34 bees from 17 colonies) tested positive for SINV-3 by RT-PCR. For each assay, the accompanying positive control (from *S. invicta*) amplified as expected.

GENBANK DATABASE SEARCHES

Extensive searches of GenBank Apoidea and Insecta-limited databases, based on the SINV-3 genome and fused polyprotein sequences, failed to detect SINV-3 in any other host. A small number of sequences with limited identity (~3%) to SINV-3 were found in *A. mellifera* and other Apoidea TSA databases. However, these sequences were determined to be much more closely related to members of the families Dicistroviridae and Iflaviridae than to SINV-3. SINV-3 has some characteristics consistent with Caliciviridae and recently has been proposed to comprise a unique family, the Solinviviridae (Valles et al. 2016).

LABORATORY INFECTION TEST

The inoculum contained 3.3×10^7 SINV-3 genome equivalents per µL of sugar water. Inoculated bee groups consumed an average of 1.1 ± 0.1 mL of the inoculum (= 3.63×10^{10} SINV-3 genome equivalents). The inoculum liquid was removed after 6 h for 5 of 6 groups but the bees from Colony A required 19 h to consume 1 mL of the inoculum. Consumption rates for the sugar water preparations in the negative control and standard groups were similar to those in the inoculated groups, again with bees from Colony A taking longer to consume 1 mL of their sugar water than bees from the other colonies.

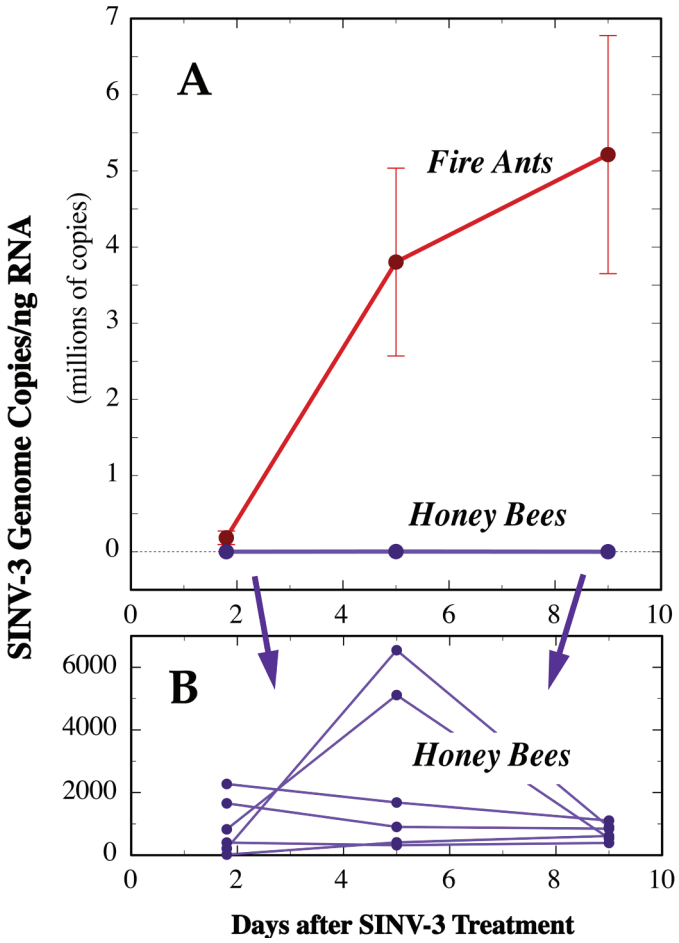

Fig. 1. A) Comparison of SINV-3 genome copies per nanogram of RNA in red imported fire ant colonies and honey bee groups inoculated with SINV-3 shown in days since being inoculated (N = 6 samples). Note that the Y-axis is in millions of copies. Error bars, where visible, show the standard error of the mean. **B)** Y-axis of top graph expanded by 1,000 times to show mean SINV-3 genome copies found in each of the 6 inoculated honey bee groups graphed over time (N = 3 bees per group).

Cups containing the SINV-3 inoculum were removed from the 6 ant colonies at 19 h, after all the bee groups had consumed at least 1 mL of bait. The ant colonies consumed only 0.5 ± 0.1 mL of the inoculum (= 1.65×10^{10} SINV-3 genome equivalents) or less than half the amount consumed by the bees despite similar group weights (3.7 g bees, 3.9 g ants). A large fraction of the inoculum removed by the ants was not consumed because it could be seen wicked into trash and deposited around the nest box. In contrast, the bees appeared to ingest all of the inoculum they collected. The net result was that each of the 30 bees received an average of about a billion genome equivalents, although there was certainly considerable variation among individuals. In contrast, each ant received an average of somewhat less than 4 million genome equivalents (assuming about 1,000 ants per g).

Despite the enormous quantities of SINV-3 consumed by both bees and ants, the quantity of virus detected in fire ants and honey bees differed dramatically over the course of the experiment (Fig. 1). In fire ants, mean viral copies per ng of RNA ± SE increased from $1.84 \times 10^5 \pm 0.89 \times 10^5$ (day 2 or 43 h) to $3.80 \times 10^6 \pm 1.23 \times 10^6$ (day 5) to $5.21 \times 10^6 \pm 1.56 \times 10^6$ (day 9). In comparison, equivalent values for the bees were only 898 ± 363, 2,490 ± 1,090, and 733 ± 107 for days 2, 5, and 9, respectively (Fig. 1), values that are congruent with external contamination and/or residual inoculum in the gut.

The mean number of copies of SINV-3 declined in 3 bee groups from day 2 to day 5 and increased in the other 3 groups (Fig. 1B). From day 5 to day 9, the mean number declined in 4 groups, increased in 1 group, and remained about the same in 1 group. However, the mean number of viral copies detected for days 2, 5, and 9 (Fig. 1B) did not differ statistically (randomized block ANOVA, $F_{2,10} = 2.09$, $P = 0.17$) because of high within-group variability probably caused by some bees ingesting larger volumes of the inoculum than other bees.

The SINV-3 capsid protein was detected in 5 of 6 fire ant colonies by 2 d after introduction of the inoculum. Strong bands were detected in all 6 of the inoculated fire ant colonies on day 5 and again on day 9 (Fig. 2), thus confirming that the inoculum was infectious and the virus was replicating and being assembled in the fire ants. In contrast, the capsid protein was not detected in any of the SINV-3 inoculated bee samples tested, whether at 2, 5, or 9 d (Fig. 2).

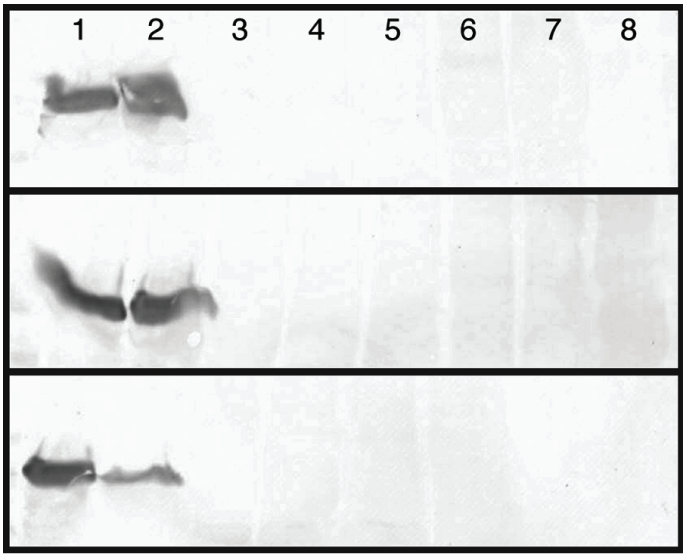

Fig. 2. Western blot analysis for SINV-3 capsid protein 9 d after inoculating 6 groups of honey bees and 6 fire ant colonies. Lanes 1 and 2 show positive detection of capsid proteins in all 6 inoculated fire ant colonies (shown in 3 rows). Lanes 3 to 8 show negative tests for 18 bees (3 from each of the 6 groups inoculated with SINV-3).

Average bee mortality in the SINV-3 inoculated groups, the negative control groups, and the standard groups did not differ at 11 d (5.0 ± 1.5 SE, 4.0 ± 1.6, and 3.8 ± 0.9, respectively; $F_{2,10} = 0.418$, $P = 0.670$) or before.

Discussion

Previous reports have shown that SINV-3 is very effective at causing significant mortality in laboratory-reared fire ant (*S. invicta*) colonies (Valles et al. 2013; Valles & Porter 2015). Thus, the virus appears to hold considerable promise as a classical biological control agent against fire ant populations in regions where the virus is absent, for example, many of the islands of the West Indies, which have been shown to be largely devoid of natural enemies (Valles et al. 2015).

Whereas previous studies have shown that SINV-3 is host specific to adult fire ants (Valles et al. 2014b) in the South American *saevissima* complex (Porter et al. 2013, 2015b, 2016), an abundance of caution and recent evidence suggesting that ants can serve as pathogen reservoirs for honey bee colonies (Levitt et al. 2013; Sebastien et al. 2015) prompted us to challenge adult honey bees directly to determine whether they could support replication of SINV-3 under laboratory conditions. Although the honey bees had been exposed to extreme quantities of SINV-3 (~1 billion genome equivalents per bee), we were able to detect only small quantities (10^{-5} to 10^{-4}% of the inoculating dose) of virus in treated bees (Fig. 1). The small amount of virus detected in the bees probably resulted because a portion of the initial inoculum was retained in the gut, a possibility made more likely because our hoarding cages did not allow the bees to fly out of the hive to defecate as would normally occur. External contamination from the inoculum and feces in the cage are also likely sources for the small amounts of virus detected. We conclude that no virus replication occurred because we were not able to detect the presence of the capsid protein (Fig. 2) and because quantities of the virus did not increase over time (Fig. 1B).

In contrast, the fire ant positive control colonies, which also had been inoculated with SINV-3, showed rapid production of newly synthesized virus (within 2 d of exposure) and dramatic increases in abundance over time based on both qPCR and capsid detection data (Figs. 1 and 2), results that are consistent with those of previous studies (Valles et al. 2014b; Valles & Porter 2015).

Furthermore, direct examination of field-collected bees from areas in which fire ants are infected with SINV-3 also proved negative for the presence of SINV-3, as did searches of GenBank sequences associated with honey bees, other Apoidea, and Insecta generally.

We demonstrated that adult honey bees are not suitable hosts for SINV-3 and that SINV-3 sequences were not found in honey bee TSA databases. Nevertheless, some positive-strand RNA viruses exhibit high mutation rates, which can result in expanded host ranges (Longdon et al. 2014). Indeed, RNA viruses are the most common source of emerging and re-emerging infectious diseases (Schneider & Roossinck 2001; Woolhouse et al. 2005). However, expansion of host range would be most likely to occur with RNA viruses that already have a broad host range (Woolhouse et al. 2005), a characteristic that clearly does not apply to SINV-3, or among closely related hosts (Woolhouse et al. 2005) like native fire ants. Organisms consuming fire ants or having extensive interactions with fire ants might also be subject to a host shift. Honey bees do not consume fire ants, but fire ant colonies and honey bee hives can sometimes be found in close proximity to each other (Deslippe & Melvin 2001). Nevertheless, despite proximity and sympatry on 2 continents for some decades, SINV-3 has not been identified in honey bees. Similarly, SINV-3 does not appear to have jumped

to native North American fire ants or thief ants despite their close relationship and sympatry (Porter et al. 2013, 2015b).

Finally, it is worth noting that no known honey bee viruses are members of Solinviviridae, the proposed family containing SINV-3; furthermore, the limited information that is available about Solinviviridae indicates that members of this family probably do not have broad host ranges (Valles et al. 2016).

Based on 1) laboratory infection tests with ants and honey bees, 2) direct tests of honey bees from the field, 3) searches of GenBank, and 4) the relatively distant relationship of SINV-3 with known honey bee viruses, we conclude that field release of SINV-3 as a biocontrol agent against red imported fire ants in regions without this virus would pose little or no threat to honey bees.

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