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Multiple virus infections occur in individual polygyne and monogyne *Solenopsis invicta* ants

Clare Allen, Steven M. Valles *, Charles A. Strong

USDA- ARS, Imported Fire Ant and Household Insects Research Unit, 1600 SW 23rd Drive, Gainesville, FL 32608, USA

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ABSTRACT

Concurrent infections of *Solenopsis invicta* colonies with *S. invicta* virus 1 (SINV-1), SINV-2, and SINV-3 has been reported. However, whether individual ants were capable of supporting multiple virus infections simultaneously was not known, nor whether the social form of the colony (polygyne or monogyne) had an influence on the occurrence of multiple infection rates in individual ants. *S. invicta* field populations were sampled sequentially to establish whether multiple virus infections co-occurred in individual worker ants. In addition, the intra-colony virus infection rates were compared in monogyne and polygyne field colonies to determine whether social form played a role in the viral infection prevalence. All combinations of virus infection (SINV-1, SINV-2, or SINV-3 alone, SINV-1 & SINV-2, SINV-1 & SINV-3, SINV-2 & SINV-3, and SINV-1, SINV-2 & SINV-3) were detected in individual worker ants as well as queens in the field. Thus, individual *S. invicta* ants can be infected simultaneously with all combinations of the *S. invicta* viruses. Colony social form did have an influence on the intra-colony prevalence of multiple *S. invicta* virus infections. Polygyne colonies exhibited significantly greater intra- and inter-colony single and multiple virus infections compared with monogyne colonies.

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1. Introduction

The red imported fire ant, *Solenopsis invicta*, was introduced into the USA in the 1930s near Mobile, Alabama (Callcott and Collins, 1996). Since that time, *S. invicta* has expanded its range to include most of the southern tier of the USA, largely concentrated from Texas to Florida, but also found as far west as California and north to Virginia (Allen et al., 2010; Williams et al., 2001). *S. invicta* has demonstrated exceptional invasive capacity with recent introductions in Australia (McCubbin and Weiner, 2002) and China (Wong and Yuen, 2005). *S. invicta* exists in two distinct social forms, polygyne and monogyne, with polygyne colonies containing multiple fertile queens while monogyne colonies contain a single fertile queen.

Although insecticides are effective against *S. invicta* populations, biological control is considered the most sustainable method of control for this pest in the USA (Porter et al., 1997). Therefore, a great deal of research effort has focused on discovery, characterization, importation, and development of biologically-based agents (Williams et al., 2003). In an effort to determine whether any viruses infect *S. invicta*, a metagenomics approach was employed (Valles et al., 2008) and revealed three RNA viruses, *S. invicta virus* 1 (SINV-1 (Valles et al., 2004)), SINV-2 (Valles et al., 2007a) and SINV-3 (Valles and Hashimoto, 2009). All are positive sense, single-stranded RNA viruses with monopartite genomes, but each is phylogenetically distinct. The International Committee on Taxonomy of Viruses has formally placed SINV-1 in the *Dicistroviridae* (Carstens and Ball, 2009), but neither SINV-2 nor SINV-3 has received formal taxonomic placement. Phylogenetic analysis of the RNA-dependent RNA polymerase (RdRp) region of the SINV-3 genome showed a relationship with Kelp fly virus (KFV), which is also currently unclassified (Valles and Hashimoto, 2009). Similar analysis of the SINV-2 RdRp and capsid protein regions revealed that this virus is phylogenetically distinct (Valles et al., 2007a). SINV-1 and SINV-2 appear to cause persistent, asymptomatic infections resulting in no discernable disease symptoms among fire ant colonies in the field. Conversely, SINV-3 is associated with significant mortality among *S. invicta* colonies (Valles and Hashimoto, 2009).

As these are recent discoveries, descriptive and hypothesis-driven research is continuing to develop an understanding of the relationship between the fire ant host and these viruses. Multiple infections of *S. invicta* colonies with SINV-1, -2, and -3 have been demonstrated (Valles et al., 2009). However, whether individual ants are capable of supporting multiple virus infections simultaneously is not known, nor whether the social form of the colony (polygyne or monogyne) has any influence on multiple virus infection rates in individual ants. Because all three viruses exhibit a tissue tropism for gut epithelial cells (Hashimoto and Valles, 2007, 2008; Valles and Hashimoto, 2009), we hypothesized that

* Corresponding author. Fax: +1 352 374 5818.
E-mail address: steven.valles@ars.usda.gov (S.M. Valles).

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competition for these cells may preclude multiple infections within individual ants.

In the USA, polygyne fire ant colonies generally exhibit lower intra-colony genetic relatedness among nest mates (Keller, 1995) while monogyne colonies exhibit high levels of genetic relatedness (Ross et al., 1996). Among social insects, genetic relatedness among nest mates may influence a colony's ability to resist pathogen infection, the genetic diversity—pathogen resistance hypothesis (Schmid-Hempel, 1998; Sherman et al., 1988). Reber et al. (2008) and Ugelvig et al. (2010) recently provided empirical support for this hypothesis in two ant species, *Formica selysi* and *Cardiocondyla obscurior*, respectively. In both ant species, it was shown that reduced intra-colony genetic diversity compromised the ability of a colony to resist parasites. Similarly, genetically diverse honeybee colonies exhibited a lower variance in disease prevalence compared with genetically related colonies which may prevent severe infections from occurring (Tarpy, 2003; Tarpy and Seeley, 2006).

To address these relationships in *S. invicta*, field populations were examined to first establish whether multiple virus infections were supported in individual worker ants. Subsequently, intra-colony virus infection rates through the examination of workers and queens were compared in both monogyne and polygyne colonies to determine whether social form influenced the intra-colony virus infection rate.

2. Materials and methods

2.1. Multiple virus infections (worker ants)

The first objective was to determine whether individual worker ants were capable of being infected with more than one virus simultaneously. In order to increase the chance of identifying multiple virus infections in individuals, field nests were sampled and examined to identify those exhibiting multiple virus infections. Worker ants were sampled between May 2009 and March 2010 from *S. invicta* colonies from field locations in Gainesville, FL, by plunging a 20 ml glass scintillation vial into a mound and collecting ants that fell into the vial. These nests were marked with surveyor flags. Total RNA was extracted from pooled groups of 10–20 ants from these nest samples with Trizol according to the manufacturer's instructions (Invitrogen, CA). The presence of SINV-1, SINV-2, and SINV-3 was determined by conducting multiplex RT-PCR (Valles et al., 2009). Colonies with dual or triple virus infections were revisited to collect additional samples of ants using the scintillation vial method. Total RNA was extracted from individual worker ants (7–15) from these colonies and each examined by multiplex RT-PCR for the presence of the viruses. Previous studies have indicated that the sensitivity for this assay is as low as 500 genome copies of the virus (Valles et al., 2009). No attempt was made to determine the social form of the colony for this experiment. Sequential analysis technique was employed for the individual sampling of multiple virus infections using the pre-defined stopping rule of positive virus detection. In other words, once each multiple infection combination (SINV-1 alone, SINV-2 alone, SINV-3 alone, SINV-1 & SINV-2, SINV-1 & SINV-3, SINV-2 & SINV-3, and SINV-1, SINV-2 & SINV-3) was observed, sampling was terminated.

2.2. Multiple virus infections (queens)

Sequential sampling was also conducted to determine whether queens were capable of hosting multiple virus infections. Newly mated queens were collected on 18 May 2010 ($n = 50$, Waldo, FL, 29.7897, −82.1673; $n = 8$, Gainesville, FL, 29.6229, −82.3839) and 26 May 2010 ($n = 80$, Ocala, FL, 29.1905, −82.1379). Queens ($n = 10$) from polygyne colonies were collected by excavation on 8 April 2010 (Gainesville, FL). The queens were decapitated and DNA extracted from the head (Valles et al., 2002) and used to genotype the ants at the *Gp-9* locus to assign social form (Valles and Porter, 2003). The head was used to permit genotyping the queen without being influenced by the allele contributed from the male (sperm). Total RNA from the corresponding thorax/abdomen from the queens was extracted with Trizol and subjected to multiplex RT-PCR for the presence of SINV-1, SINV-2, and SINV-3 (Valles et al., 2009). Examination of individual queens for viruses was conducted until detection of all three viruses (SINV-1, -2, and -3) in a single queen was observed.

2.3. Colony social form influence on virus infections in individual worker ants

To examine whether colony social form may have influenced the intra-colony prevalence or number of viruses infecting individuals in a colony, individual worker ants were sampled from areas in which polygyne and monogyne nests were sympatric. Sites were selected at random. Four to eight nests from each location were sampled within 50 meters of one another by the scintillation vial method described above. DNA was extracted from a pooled group of 10 worker ants from each of these colonies and genotyped at the *Gp-9* locus to determine the social form (Valles and Porter, 2003). Among the sites examined, eight were identified with polygyne and monogyne colonies closely sympatric (Table 1). Individual ants ($n = 7$) from polygyne and monogyne nests from the eight locations were examined by multiplex RT-PCR (Valles et al., 2009) for the presence of SINV-1, SINV-2 and SINV-3.

2.4. Statistical analyses

The intra-colony infection rate of SINV-1, SINV-2, and SINV-3 was compared individually in polygyne and monogyne workers for each location by Student's *t*-test. In this analysis, each individual was considered an experimental unit.

To examine the possibility of viral infection being dependent on colony social form, infection data from individuals were pooled without regard for location. Individuals and colonies were categorized as being uninfected (no virus detected), having a single virus infection (only one virus detected), or having a multiple virus infection (two or more viruses detected simultaneously). These data were compared by Fisher's Exact test in a 3×2 factorial which tested the null hypothesis that virus infection level (uninfected, single, or multiple) was independent of colony social form (monogyne, polygyne).

3. Results and discussion

3.1. Multiple virus infections

Sequential sampling of worker ants from the field, without regard for social form, revealed that SINV-1, SINV-2 and SINV-3 can co-infect individual worker ants (Fig. 1). All combinations of virus infection (SINV-1, SINV-2, or SINV-3 alone, SINV-1 & SINV-2, SINV-1 & SINV-3, SINV-2 & SINV-3, and SINV-1, SINV-2 & SINV-3) were detected in individual worker ants. Similarly, queens of both social forms (monogyne and polygyne) also exhibited co-infection with SINV-1, SINV-2, and SINV-3 (Fig. 1). Thus, individual *S. invicta* ants can be infected simultaneously with all combinations of the *S. invicta* viruses. This result was somewhat anticipated because co-infection of honey bee queens by multiple single-stranded RNA viruses was observed in up to 93% of individual bees assayed (Chen et al., 2005; Evans, 2001). Because SINV-1, SINV-2 and SINV-3 are thought to be vertically transmitted (Hashimoto and Valles,

Table 1
Collection dates, locations and social form of the *S. invicta* colonies sampled to examine whether colony social form influences the intra-colony prevalence or number of viruses infecting individuals in a colony.

Location (designation)	Date of collection	Latitude, longitude	Social form (n)[a]
Fred Bear Road, Gainesville, FL (1MP)	14 September 2010	29.6122, −82.3831	Monogyne (2), Polygyne (2)
US 441, Micanopy, FL (2MP)	16 September 2010	29.4968, −82.2962	Monogyne (2), Polygyne (2)
SW 23rd Drive, Gainesville, FL (3MP)	20 September 2010	29.6341, −82.3612	Monogyne (2), Polygyne (2)
County Road 26, Windsor, FL (4MP)	13 October, 2010	29.6995, −82.1809	Monogyne (2), Polygyne (2)
US 441, Bellview, FL (5MP)	13 October, 2010	29.0719, −82.0721	Monogyne (1), Polygyne (2)
Newberry Road, Newberry, FL (6MP)	October 2010	29.6546, −82.5357	Monogyne (2), Polygyne (1)
Archer Road, Gainesville, FL (7MP)	19 October, 2010	29.5833, −82.4496	Monogyne (2), Polygyne (2)
County Road 24, Otter Creek, FL (8MP)	1 November, 2010	29.3059, −82.8064	Monogyne (2), Polygyne (2)

[a] Sample size represents the number of colonies of each social form sampled from that location.

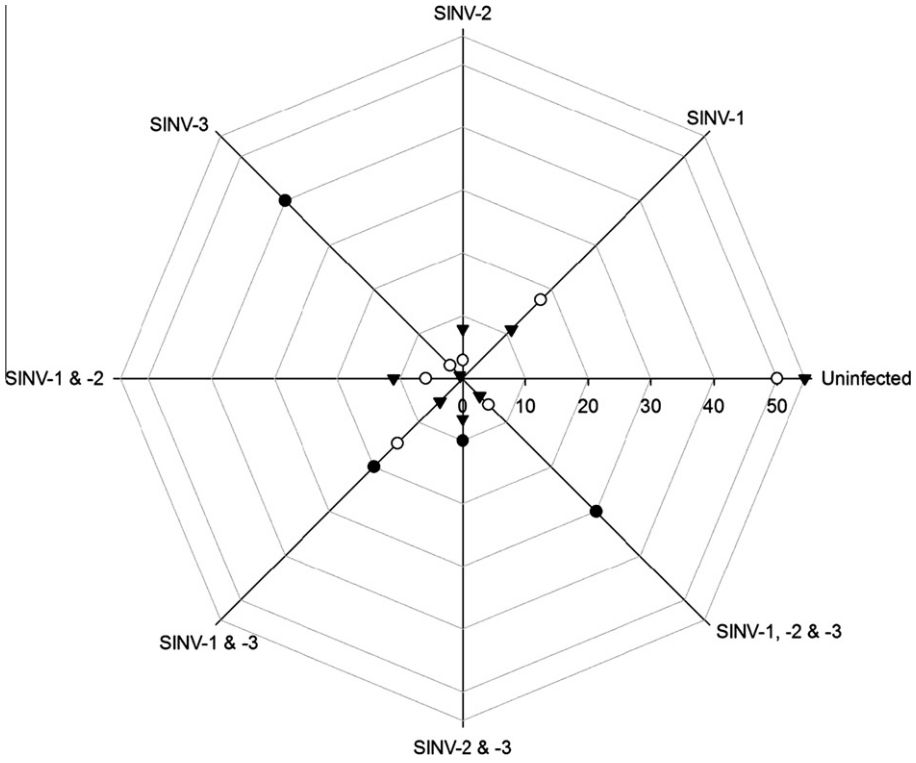

Fig. 1. Radial plot illustrating virus infections co-occurring in individual worker ants (▼), polygyne (●) and monogyne (○) queens. Point values represent the proportion of workers (n = 154), polygyne queens (n = 10), and monogyne queens (n = 34) in each infection category. Sampling was conducted sequentially and terminated once individual workers and queens were identified with every combination of virus infection. Horizontal axis represents the the proportion of *S. invicta* individuals.

2008; Valles and Hashimoto, 2009; Valles et al., 2004), one possible mode of virus dissemination to incipient colonies likely occurs via infected newly-mated queens.

3.2. Colony social form and virus infections in individual worker ants

Thirty colonies of *S. invicta* were sampled from eight locations around North Central Florida, 15 polygyne and 15 monogyne (Table 1). The collection dates (September–November 2010) were chosen specifically because the prevalence of SINV-1 and SINV-3 in *S. invicta* has been shown to be related to temperature (Valles et al., 2010; Valles et al., 2007b). SINV-1 is most prevalent during the warmer periods of the year while SINV-3 during cooler times. The autumn sampling provided a compromise and the best opportunity to find both virus infections co-occurring. SINV-2 prevalence appears to be independent of temperature (Valles et al., 2010).

Single viral infection rates in individual worker ants ranged from 0 to 92.9% (Table 2). SINV-1 was the most common virus detected in individual worker ants with an overall mean infection rate of 48% (monogyne and polygyne data combined), while the overall prevalence of SINV-2 and SINV-3 was less than 10% for each virus (Table 2). The SINV-1 infection rate for individual workers was generally similar between sympatric polygyne and monogyne colonies, with the exception of sites 6MP, 7MP, and 8MP (Table 2). These three sites exhibited higher SINV-1 infections in individual ants from polygyne colonies. Conversely, detection of SINV-2 and SINV-3 was irregular and did not appear to coincide with location or colony social form. Combined data from all sites (i.e., the grand mean) revealed an individual worker SINV-1 infection rate significantly higher in polygyne ants compared with their monogyne counterpart. SINV-3 infection rate was significantly higher in polygyne colonies from locations 4MP and 6MP. However, no significant differences in SINV-2 and SINV-3 infection rates were observed between polygyne and monogyne ants when the data from all the locations were combined. The rate of virus infection observed in individual ants in polygyne and monogyne colonies was similar to the intra-colony virus infection rates reported previously (Valles et al., 2010).

SINV-1 was 7-times more prevalent in individual ants than SINV-2 or SINV-3, which was also similarly reported at the colony level (Valles et al., 2010). Furthermore, 93% of the polygyne colonies were infected with SINV-1 compared with only 73% of

Table 2
Comparison of SINV-1, SINV-2 and SINV-3 infection rates in individual *S. invicta* workers of each social form at each location in which monogyne and polygyne colonies were sympatric.

Location designation	Individual infection rate (% ± SEM)					
	SINV-1		SINV-2		SINV-3	
	Monogyne	Polygyne	Monogyne	Polygyne	Monogyne	Polygyne
1MP	78.6 ± 11.4	92.9 ± 7.1	0	7.1 ± 7.1	0	0
2MP	57.1 ± 13.7	57.1 ± 13.7	21.4 ± 11.4	0	7.1 ± 7.1	0
3MP	64.3 ± 13.3	64.3 ± 13.3	0	0	0	0
4MP	42.9 ± 13.7	50.0 ± 13.8	0	0	0*	42.9 ± 13.7
5MP[a]	57.1 ± 20.2	42.9 ± 13.7	14.3 ± 14.3	21.4 ± 11.4	0	0
6MP[b]	0*	85.7 ± 14.3	0	85.7 ± 14.3	7.1 ± 7.1*	57.1 ± 20.2
7MP	14.3 ± 9.7*	64.3 ± 13.3	0		0	0
8MP	0	21.4 ± 11.4	0		14.3 ± 9.7	0
Grand mean (n = 105)	38.1 ± 4.8*	58.1 ± 4.8	3.8 ± 1.9	9.5 ± 2.9	3.8 ± 1.9	9.5 ± 2.9

Mean proportions for each location and viral infection with an asterisk indicates statistical significance by Student's t-test ($p < 0.05$).
[a] Single monogyne colony.
[b] Single polygyne colony.

monogyne colonies examined. Although the level of intra-colony virus infection undoubtedly depends on a number of factors (e.g., time of infection, virus isolate, environment, etc.) for which we did not control, it was surprising that none of the colonies exhibited an infection rate of 100%. SINV-1, SINV-2 and SINV-3 exhibit tissue tropism toward the midgut (Hashimoto and Valles, 2007; Hashimoto and Valles, 2008; Valles and Hashimoto, 2009) from which virus particles are thought to be shed into the gut lumen and, presumably, distributed throughout the colony by trophallaxis (Oi and Valles, 2009). Thus, trophallaxis was expected to result in efficient colony-wide dissemination and ultimately very high intra-colony infection rates. However, social insects are known to employ a sophisticated array of individual and communal barriers to minimize the establishment and spread of parasites and pathogens within the colony (Evans and Spivak, 2010) which may explain why a 100% SINV-1 intra-colony infection rate was not observed.

Monogyne *S. invicta* exhibited significantly greater proportions of individuals and colonies without virus infection compared with polygyne colonies (Fig. 2). The occurrence of single and simultaneous multiple virus infections were significantly greater in polygyne colonies and individuals compared with monogyne colonies (Fig. 2). Indeed, 93% of the polygyne colonies were infected with at least one virus compared with only 73% of monogyne colonies. In addition, a correspondingly higher proportion of individuals in polygyne colonies (60%) were infected with at least one virus while only 41% of individuals from monogyne colonies were infected. Valles et al. (2010) similarly reported a higher incidence of multiple infections of viruses, the microsporidian *Kneallhazia solenopsae*, and *Pseudacteon* parasitoid flies in *S. invicta* polygyne colonies compared with monogyne colonies.

Genetic diversity among individuals in social insect colonies has been shown to afford resistance to parasitism (Reber et al., 2008; Tarpy, 2003; Ugelvig et al., 2010; van Baalen and Beekman, 2006). Because polygyne fire ant colonies exhibit greater intra-colony genetic diversity compared with monogyne colonies, we expected polygyne fire ant colonies to exhibit lower intra-colony virus infection rates. However, the intra-colony virus infection rate was higher in polygyne fire ant colonies compared with monogyne colonies. Although our results do not appear to support the genetic diversity–parasitism resistance hypothesis, we did not conduct a properly controlled study to specifically examine this relationship. In *S. invicta*, polygyne colonies exhibit characteristics that may be expected to facilitate pathogen infection in that they are generally longer-lived, possess larger colonies, and are socially open (they continually accept and contain multiple unrelated queens (Tschinkel, 2006)) compared with monogyne colonies. In this regard, our data support the contention of van Baalen and Beekman (2006) that heterogeneous colonies have a larger suite of parasites

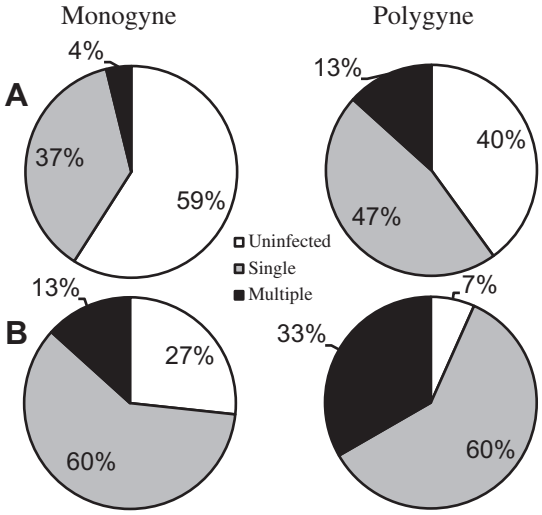

Fig. 2. Pie charts comparing *S. invicta* virus infections in monogyne and polygyne *S. invicta*. (A) Proportion of individuals (n = 105 for each social form) from all colonies sampled (n = 15 for each social form) in which no virus infection was detected (uninfected), a single virus was detected (single), or more than one virus was detected (multiple). Fisher's Exact test revealed significance ($p < 0.05$) indicating that individual virus infection status was dependent on colony social form. (B) Proportions of colonies (n = 15 for each social form) in which no virus infection was detected (uninfected), a single virus was detected (single), or more than one virus was detected (multiple). Fisher's Exact test revealed significance ($p < 0.05$) indicating that colony virus infection status was dependent on colony social form.

that can infect them. Indeed, these authors further conclude that the advantages of genetic diversity may not be as important in conferring parasite resistance as previously thought.

This is the first report demonstrating multiple virus infections in individual fire ants. Although multiple virus infections have been reported in the honey bee (Chen and Siede, 2007), the results in ants are somewhat surprising because SINV-1, SINV-2 and SINV-3 exhibit tissue tropism toward the midgut epithelial cells of adults and larvae of *S. invicta* (Hashimoto and Valles 2007, 2008; Valles and Hashimoto 2009). Thus, competition for the same cell type would be expected to preclude multiple infections as suggested by Flegel (2007) in that a single virus inside a cell could potentially interfere with the entry and replication of another virus. Although there is no current evidence to show that a single *S. invicta* cell possesses concurrent virus infections, it has been previously demonstrated that individual cells are able to accommodate multiple viruses (Anderson, 1942; Reissig, 1959). Anderson and Gibbs (1988) reported that Sacbrood virus and Black queen cell virus replication in honey bees was suppressed by activation of Kashmir bee virus.

Multiple *S. invicta* virus infections were not as common as single virus infections suggesting that this situation may not be the most favorable environment for virus development or that the probability of an ant encountering multiple viruses is lower than encountering a single virus. Another consideration of multiple infections is the potential exchange or intermixing of portions of the virus genomes. Positive strand RNA viruses replicate autonomously in the cytoplasm of the host cell and multiple genome copies (positive and negative forms) would be present. Furthermore, template switching, a recombination mechanism exhibited by positive strand RNA viruses, (Freistadt et al., 2007) may facilitate virus evolution possibly leading to species creation. Thus, co-occurrence of the *S. invicta* viruses within a host cell could lead to the evolution of new virus species by the template switching mechanism. Indeed, intra-virus recombinants of Israeli acute paralysis virus have been reported in the honey bee host (Maori et al., 2007) and Acute bee paralysis virus, Israeli acute paralysis virus, and Kashmir bee virus are all recent descendents or variants of one another (Baker and Schroeder, 2008; de Miranda et al., 2010). Moore et al. (2011) have demonstrated inter-virus recombinant events between Deformed wing virus and Varroa destructor virus 1. A more thorough understanding of the effects of multiple virus infections in individual fire ants and corresponding differences in virus susceptibility by each social form will facilitate efficient use of biological control agents to control this invasive species.

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