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Laboratory host specificity testing of the fire ant microsporidian pathogen *Vairimorpha invictae* (Microsporidia: Burenellidae)

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ABSTRACT

The host specificity of *Vairimorpha invictae*, a microsporidian pathogen of fire ants in South America, was assessed in the laboratory. Species evaluated included the tropical fire ant, *Solenopsis geminata*, the southern fire ant, *Solenopsis xyloni*, and the Argentine ant, *Linepithema humile*. The two fire ant species are native to North America. The Argentine ant is a widespread, exotic species that co-occurs with the native North American fire ants as well as with the red imported fire ant, *Solenopsis invicta*, and the black imported fire ant, *Solenopsis richteri*, in the US. Inoculations of *V. invictae*-infected *S. invicta* brood to laboratory colonies did not result in any infections of *S. geminata*, *S. xyloni*, or *L. humile*, while 60% of the *S. invicta* colonies developed infections. *V. invictae* was not detected in smaller groups of *S. geminata* and *S. xyloni* larvae that were tended by *V. invictae*-infected adult, *S. invicta* workers, but was detected in 40% of the *S. invicta* larval groups tended by infected workers. This was the first report of *V. invictae* transmission to larvae by infected adult worker ants. Exposure to *V. invictae* by contact with infected brood and workers partially emulated possible field interactions between infected and uninfected ant species. These results are congruent with previous field surveys which indicate that the host range of *V. invictae* is limited to fire ants of the *Solenopsis saevissima* species group.

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1. Introduction

Red imported fire ants, *Solenopsis invicta* Buren, are stinging, invasive pest ants in the United States of America (US), causing an estimated annual loss of $6.3 billion (Lard et al., 2006). In their native range they are infected with *Vairimorpha invictae*, a microsporidian pathogen of fire ants that was first described by Jouvenaz and Ellis (1986) from *S. invicta* collected in Brazil. It is an obligate, intracellular parasite that infects fat body cells and produces two morphologically distinct spores (Jouvenaz and Ellis, 1986). Infected *S. invicta* workers died sooner than uninfected ants in the laboratory (Briano and Williams, 2002). *V. invictae* field infections alone and in combination with another microsporidan pathogen of fire ants, *Kneallhazia* (=*Thelohania*) *solenopsae* Knell, Allen and Hazard, are associated with dramatic declines (53–100%) in localized *S. invicta* populations in Argentina (Briano, 2005).

Currently, *V. invictae* is not known to occur in the US (Oi et al., 2005; Oi and Valles, 2009) and the microsporidium is being evaluated for release in the US as a biological control agent of *S. invicta*. In assessing its host specificity, examinations by microscopy of ants collected at baits and from nests in Argentina and Brazil by Briano et al. (2002) revealed infections of *V. invictae* in *S. invicta, Solenopsis richteri* Forel, and *Solenopsis macdonaghi* Santschi. Infections were most prevalent in *S. invicta*, and *V. invictae* infections were not observed in 10 non-*Solenopsis* ant genera. Similarly, infections were not detected by PCR assay in 235 non-ant arthropods and 509 non-*Solenopsis* ants collected at three *V. invictae* infected sites in Argentina (Porter et al., 2007). Here we report the results of laboratory inoculations with *V. invictae* in two fire ant species native to North America, the tropical fire ant, *Solenopsis geminata* (F.), and the southern fire ant, *Solenopsis xyloni* McCook. In addition, we inoculated laboratory colonies of the Argentine ant, *Linepithema humile* (Mayr), a species that co-occurs with the two native North American fire ants as well as with *S. invicta* and *S. richteri* in both the US and in Argentina (Suarez et al., 2001; Streett et al., 2006; Tschinkel, 2006; LeBrun et al., 2007). The Argentine ant is an invasive, exotic species from South America that entered the US in the late 1800s (Newell, 1908). This species belongs to the subfamily Dolichoderinae while *Solenopsis* fire ants are in the subfamily Myrmicinae.

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2. Materials and methods

2.1. Whole colony inoculations

Colonies of *S. xyloni*, *S. geminata*, and *S. invicta* were established in the laboratory to determine their susceptibility to *V. invictae*. *S. xyloni* colonies were collected in Fresno County, California and transported to the quarantine facility at the USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida, in 2004. *S. geminata* colonies were collected in 2003–2005 in Alachua County, Florida, and *S. invicta* colonies were reared from newly-mated queens collected in 2004, from Alachua County. Colonies were reared without soil in nest cells, following procedures adapted from imported fire ant rearing methods of Banks et al. (1981). All colonies were reared to what was considered to be healthy and large in size, containing at least one queen and having initial colony sizes ranging from 1000 adult workers and 7 ml of brood to 15,000 workers and 35 ml of brood (Table 1).

Colonies were inoculated with brood from five *V. invictae*-infected *S. invicta* colonies collected in February 2005 near San Javier, Santa Fe province, in eastern Argentina, one of the sites with the highest infection prevalence (Briano et al., 2006). These colonies were hand carried to the ARS quarantine in Gainesville with proper export/import permits. *V. invictae*-infected brood (referred to as inocula) consisting of an arbitrary mixture of primarily live larvae, pupae, and some eggs, were placed adjacent to nest cells. Workers from the recipient colony carried the inocula into the nest. Inocula had an average infection prevalence of 96% (range 80–100%) based on individual wet mounts of pupae ($n = 10$ per colony) examined for spores by microscopy. The method of using infected brood placed adjacent to nest entrances has been used to successfully transmit *V. invictae* to uninfected *S. invicta* colonies (Oi et al., 2005). Brood was not added to control colonies.

Infections were determined from samples of pupae collected at 2 or 4 week intervals 8–24 weeks after inoculation. Wet mounts of macerated groups of pupae were examined for *V. invictae* spores by phase-contrast microscopy or by PCR assay (Oi et al., 2005; Valles et al., 2004) for each sample date. Samples usually consisted of a minimum of 10 pupae per inoculated colony per sampling date resulting in an average total of 44 (range, 30–50), 35 (range, 14–50), and 66 (range, 30–90) pupae per colony being evaluated for infection for *S. xyloni*, *S. geminata*, and *S. invicta*, respectively. Some samples had less than 10 pupae due to low levels of brood. *S.invicta* colonies had more pupae sampled relative to the other species because their larger colonies allowed more frequent sampling. Control colonies were sampled at 12 and 20 weeks for a total of 20 pupae per colony. The sampling dates provided ample time for adult eclosion of inocula (Porter, 1988) and thus prevented the confounding of pupal samples. Pupae were used to determine infections because they are often the most prevalent immature stage that can be recovered from infected colonies (DHO personal observation) and the removal of larvae can reduce the fecundity of *S. invicta* queens (Tschinkel, 1995).

The number of workers, volume of brood, and the presence of live queens were visually estimated at 1, 2, or 4 week intervals to document the impact of infections on colonies. Brood volume and the number of adults were averaged over samplings from weeks 8 through 20, which was when changes in these parameters were observed in infected colonies. Brood and worker levels were compared by one-way analyses of variance and Tukey's HSD test (SAS Institute, 2008), among infected, inoculated uninfected, and control colonies by ant species.

Inoculations were made to small laboratory colonies of Argentine ants, which were collected in Marion County, Florida. Colonies were reared in the laboratory approximately 2.5 months before the initiation of the study. Argentine ant colonies used in the study contained an average of 500 (range, 200–1000) workers, 1.9 ml (range, 0.75–4.5) of brood, and 2 (range, 1–5) queens. *S. invicta* colonies, used as positive or negative controls, contained an average of 80 (range, 35–150) workers, 1.5 ml (range, 0.5–2.5) of brood, and 1 queen. These small colonies were reared in nesting tubes made from glass test tubes (16 mm diameter × 150 mm length) half filled with water retained behind a cotton plug topped with a ≈1 cm layer of dental stone (Castone®, Dentsply International, St. Louis, MO). Inocula were obtained from four *V. invictae*-infected colonies collected in August 2007 near San Javier in Argentina. Based on the number of infected pupae per sample of 10 pupae per colony, 52% (weighted average, range 30–60%) of the inocula was infected. Colonies were inoculated with 4 g of an arbitrary mixture of *S. invicta* brood consisting mainly of live pupae and 4th instar larvae, which was poured into each nest tube. Brood was not added to control colonies. The number of infected colonies was determined through PCR or microscopy of pupae at 9.7, 10.9 (*S. invicta* only), and/or 13.1 weeks after inoculation. Approximately 10–20 pupae were collected per sample date per inoculated colony for a total of about 20–40 Argentine ant pupae and 30–60 *S. invicta* pupae per colony being tested for infection. Colonies were considered infected when *V. invictae* was detected on a minimum of two sample dates. The study was conducted at an average of 26 °C (range 25–27 °C). Larva (1st instar) to adult development time of *S. invicta* was estimated to be 38 days and egg to adult development time was 45 days at 26 °C (Porter, 1988). The majority of the inocula were 4th instars and pupae, hence inocula had sufficient time for adult emergence and would not confound the determination of infection in the *S. invicta* colonies used to confirm that inoculations could result in infections (positive controls).

2.2. Larval inoculations by tending infected workers

Groups of *V. invictae*-infected *S. invicta* workers collected from Argentina (ca. 100–125 workers/group, infection prevalence 30–80%) were given access to fifty uninfected 2nd/3rd instar and fifty 4th instar larvae of either *S. xyloni, S. geminata,* or *S. invicta*. The presence of sclerotized mandibles in 4th instars were used to distinguish them from the younger instars (Petralia and Vinson, 1979). Control groups consisted of uninfected workers of all three

Table 1
Initial colony parameters and incidence of *Vairimorpha invictae* infection among inoculated and control laboratory colonies of three *Solenopsis* fire ant species.

	Inoculated			Control[b]		
	S. invicta	*S. geminata*	*S. xyloni*	*S. invicta*	*S. geminata*	*S. xyloni*
Average (range) no. adult workers/colony	7400 (5000–10,000)	8200 (2000–15,000)	3100 (1000–10,000)	7400 (5000–10,000)	8750 (5000–15,000)	4200 (1000–15,000)
Average (range) ml brood/colony	22 (20–25)	21 (10–30)	12.8 (7–25)	23 (20–30)	22.5 (20–25)	15.8 (7–35)
Average (range) no. queens/colony	1	1	3.2 (1–10)	1	1	2.2 (1–4)
Infected no. colonies/no. inoculated colonies	3/5	0/5[a]	0/5	0/5	0/4	0/5

[a] One colony had no brood by week 8 and was dead at week 10; last PCR sample at week 7.
[b] Control colonies tested for *V. invictae* infections by PCR at weeks 12 and 20.

fire ant species tending 2nd–4th instars of their own species, with workers and larvae originating from different colonies. Workers were held in nesting tubes as described previously, but tubes were shorter in length (100 mm). Larvae were either poured into the nesting tube that contained the workers or larvae were placed in an open micro-centrifuge tube (1.5 ml) that was partially inserted into the nesting tube. With the latter method, workers carried the larvae into the nesting tube. Infected and uninfected workers were allowed to tend larvae until they developed to pupae that were at least slightly melanized, as indicated by light brown legs, darkening gaster, and off-white head color. The pupae were then collected and tested for *V. invictae* infection by PCR. In some instances pupae were either not melanized or more fully melanized with dark legs and gaster, and a gray-brown head. Worker/larvae groups were held at about 26 °C and pupae collected 20–38 days after larval introduction to workers.

3. Results

3.1. Whole colony inoculations

For the *Solenopsis* inoculations, *V. invictae* was detected in 60% of the *S. invicta* colonies. No infections were observed in *S. xyloni* and *S. geminata* colonies, and all control colonies were uninfected (Table 1). The number of workers and volume of brood averaged over weeks 8–20 after inoculation were not significantly different ($F \leqslant 1.09$; $df = 2, 7$; $P \geqslant 0.38$) among the infected inoculated colonies, uninfected inoculated colonies, and the control colonies of *S. invicta*. The period of 8–20 weeks post-inoculation was selected because it corresponded to the initial decline in brood volume in the infected colonies, but was prior to declines in the control colonies (Fig. 1A). Delay in the onset of brood decline in infected colonies has been reported by Oi et al. (2005) for *V. invictae*, as well as with *K. solenopsae* (Williams et al., 1999). Under our quarantine conditions, it also is not uncommon for uninfected field collected colonies to decline after 5 months (DHO personal observation). The number of workers and volume of brood were not significantly different between the uninfected inoculated colonies and the control colonies for *S. geminata* ($F \leqslant 1.71$; $df = 1, 6$; $P \geqslant 0.24$) and *S. xyloni* ($F \leqslant 0.14$; $df = 1, 8$; $P \geqslant 0.72$) (Fig. 1B and C).

All species of fire ants walked upon and carried the brood inocula. *S. invicta* readily carried brood into the nest cells, and with the exception of one colony, adopted the brood. Colonies of the other two species of fire ants did not adopt the brood as consistently. Within two days all the *S. geminata* and 4 of 5 of the *S. xyloni* colonies had placed the brood inocula on refuse piles.

The inoculation of Argentine ant colonies did not result in *V. invictae* infection. In contrast, 60% of the inoculated *S. invicta* colonies were infected. None of the control colonies became infected (Table 2). *S. invicta* brood added directly into the nest tubes of the Argentine ant nests facilitated handling of the inocula. Initially the Argentine ants moved out of the nest tubes as the *S. invicta*

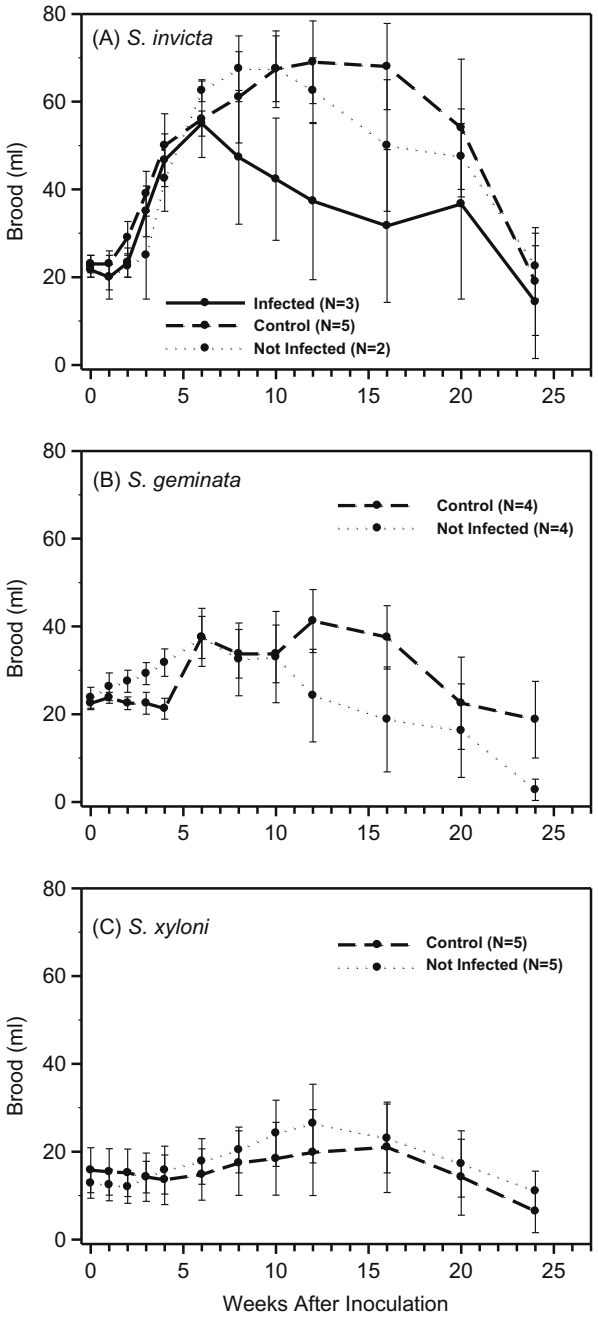

Fig. 1. Changes in mean (±SEM) brood volume per colony among *Vairimorpha invictae* infected, inoculated but not infected, and control laboratory colonies of (A) *Solenopsis invicta*, (B) *Solenopsis geminata*, and (C) *Solenopsis xyloni* after inoculations with brood from *Vairimorpha invictae*-infected *Solenopsis invicta* colonies from Argentina.

Table 2
Number of Argentine ant, *Linepithema humile*, and *Solenopsis invicta* colonies infected with *Vairimorpha invictae* that were inoculated with *Vairimorpha invictae*-infected *Solenopsis invicta* brood.

	Inoculated		Control	
	S. invicta	*L. humile*	*S. invicta*	*L. humile*
Average (range) no. adult workers/colony	88 (40–150)	500 (250–1000)	73 (35–150)	500 (350–800)
Average (range) ml brood/colony	1.4 (0.5–2.5)	1.8 (0.8–4.0)	1.5 (0.5–2.5)	2.0 (0.8–4.5)
Average (range) no. queens/colony	1 (1–1)	2.8 (2–5)	1 (1–1)	2.5 (1–5)
Infected no. colonies/no. inoculated colonies	3/5[a]	0/6	0/6[b]	0/6

[a] One inoculated colony that declined abnormally to minimal brood at week 3 was excluded from the study.
[b] One control colony tested for *V. invictae* infections by PCR at week 7, the remainder at 13 or 14 weeks after initiation of study.

brood was much larger in size than Argentine ant brood. Within a day, Argentine ants reentered their nests, and in 2 of the 6 nests, *S. invicta* brood was being tended. However, inocula eventually appeared to be discarded from all the Argentine ant nests.

3.2. Larval inoculations by tending infected workers

Infections were not detected in any of *S. xyloni* and *S. geminata* pupae that were reared by the infected worker groups ($n = 8$ and $n = 7$ groups, respectively), nor were any infections detected in the controls. *V. invictae* was present in *S. invicta* pupae in 40% (4/10) of the worker groups, indicating that transmission by infected workers was possible. Recovery of melanized pupae from larvae tended by worker ants of a different species was limited. *S. geminata* pupae were not recovered from groups tended by either infected or uninfected *S. invicta* workers. However, one group tended 2 larvae until pupation and evidently eliminated soon after. *S. xyloni* pupae were recovered from 25 and 43% of the groups, but only a total of 12 and 4 pupae were recovered from the infected and uninfected *S. invicta*, respectively. In contrast, 63–203 pupae were reared from 90–100% of worker groups tending the same species of larvae (Table 3).

4. Discussion

Laboratory inoculations of *V. invictae* did not result in infection of the fire ant congeners *S. geminata* and *S. xyloni*, or the Argentine ant. Infections were detected in 60% of the *S. invicta* colonies inoculated with brood and 40% of the pupal groups reared by infected workers (Tables 1–3), indicating the inoculation methods used were sufficient to cause infection. This is the first report of transmission of *V. invictae* by infected workers tending brood. Transmission to brood by tending infected workers has been reported for the microsporidian ant pathogen *K. solenopsae* (Allen and Knell, 1980). Only one of the three infected colonies showed a consistent reduction in brood beginning at 8 weeks after inoculation (Fig. 1), so the apparent impact was not significant. Brood reductions in the other two colonies occurred after 20 weeks, when control colonies also began to decline. This is the first report of initiating infections in large, queen-right laboratory colonies of *S. invicta* containing over 5,000 workers and 20 ml of brood. In comparison, the first successful laboratory inoculations used small, incipient host colonies that contained 30 workers and $\leqslant$1 ml of brood (Oi et al., 2005). Larger colonies were used for this study, because the *S. geminata* and *S. xyloni* colonies were mature, field collected colonies.

Human mediated transmission of *V. invictae* and *K. solenopsae* to its host *S. invicta* has not been achieved with isolated spores despite many documented attempts (Oi and Valles, 2009). However, *V. invictae* infections have been successfully transmitted by the introduction of live infected brood or infected workers that have died naturally (Oi et al., 2005), and tending of larvae by infected workers (this study). These methods involve a behavioral component, where ants somehow transfer the pathogen to uninfected ants from a different colony. In the host specificity tests, the likelihood of this transfer is decreased with the possible need for cross-fostering of brood between species. In our study, the tending and/or recovery of some *S. geminata* and *S. xyloni* pupae after introductions of larvae into *S. invicta* worker groups suggested that congeneric cross-fostering can occur, albeit at a much lower rate than within a species. This was evident by the recovery of well over five times more pupae when larvae were reared by conspecific workers (Table 3). Interspecific brood retrieval into a nest and tending has been documented for several ant species. However, alien brood survival was often temporary, the brood either being eaten or destroyed especially if not conspecific (Carlin, 1988; Hölldobler and Wilson, 1990). In our study, the transmission of *V. invictae* from tending *S. invicta* workers to conspecific larvae may have originated with spores from workers that died during the study. Dead workers were observed in pupal groups that were infected with *V. invictae*. Transmission of the virulent fat body microsporidium *Vairimorpha disparis* (Timofejeva) in gypsy moth, *Lymantria dispar* (L.), is by release of spores from larval cadavers (Goertz and Hoch, 2008). Whether the lack of infection in the two native fire ants is due to limited interspecific cross-fostering of brood or lack of physiological susceptibility cannot be distinguished in this test.

With the introductions of infected *S. invicta* brood, infection via cross-fostering to the pupal or adult stage may be less critical as transmission potentially may be initiated by the consumption of brood. Whole colony inoculations partially emulate one form of natural interference competition where a larger colony may extirpate a smaller, infected *S. invicta* colony and possibly consume infected ants and brood. Transmission of *K. solenopsae* to larger uninfected *S. invicta* colonies has been demonstrated in the laboratory when the larger colonies eliminated smaller infected, *S. invicta* colonies (Oi and Williams, 2003). Morrison (2000) demonstrated that *S. invicta* usually dominated *S. geminata* in various types of colony level interference competitions, except *S. geminata* would outcompete *S. invicta* if it was numerically superior. Argentine ants were once dominant in the states along the Gulf of Mexico in the southern US before the spread of *S. invicta* (Smith, 1936; Wilson, 1951; Wilson and Brown, 1958). While *S. invicta* generally predominate (Wilson, 1951; Glancey et al., 1976), Argentine ants can maintain populations in areas where *S. invicta* also occurs (Kabashima et al., 2007; DHO personal observation). Experimental competitions between the two species indicate that either species can survive confrontations depending on their relative colony sizes (Kabashima et al., 2007). Our data, however, did not indicate transmission of *V. invictae* to Argentine ant colonies nor to *S. geminata* or *S. xyloni* colonies when brood was used as inocula and placed near or in colonies to maximize contact.

Solter and Maddox (1998) contended that ecological, or field, host specificity is narrower than that of physiological, or laboratory, susceptibility. They showed that several species of indigenous North American lepidopteran microsporidium were never detected in sympatric field populations of gypsy moth despite physiological susceptibility of gypsy moth larvae in the laboratory. Host specificity testing of mosquito and other lepidoptera microsporidia also has indicated narrower ecological host ranges. While physiological susceptibility testing can indicate potential host range, results can be ecologically unrealistic where high spore concentrations and artificial inoculation methods may overcome natural barriers to infection (Solter and Becnel, 2007). Infections of *Burenella dimorpha*

Table 3
Number of groups with *Vairimorpha invictae* infected pupae that developed from larvae tended by infected or uninfected workers among three species of fire ants.

Tending workers	No. of infected groups/No. of larval groups among species		
	S. invicta (N)[a] [N][b]	*S. geminata* (N) [N]	*S. xyloni* (N) [N]
Infected *S. invicta*	4/10 (9) [157][b]	0/8 (0) [0]	0/8 (2) [12]
Uninfected *S. invicta*	0/10 (10) [209][b]	0/7 (0) [0]	0/7 (3) [4]
Uninfected *S. geminata*	–	0/5 (5) [63]	–
Uninfected *S. xyloni*	–	–	0/5 (5) [69]

[a] (N) = number of groups from which pupae developing from larvae were recovered.
[b] [N] = total number of pupae tested for *K. solenopsae* infection.

Jouvenaz and Hazard, a microsporidian pathogen of *S. geminata*, can be transmitted by feeding spores mixed with chicken egg yolk to colonies of the host as well as the *Solenopsis* fire ants *S. invicta*, *S. richteri*, and *S. xyloni* (Jouvenaz and Hazard, 1978). However, *B. dimorpha* infections did not persist in laboratory colonies of these species; nor was it found in *S. invicta* field colonies that coexisted with *S. geminata* (Jouvenaz, 1983). For *V. invictae*, successful laboratory inoculations by feeding isolated spores in diet substrates has not been accomplished even in its natural host (Jouvenaz and Ellis, 1986; Shapiro et al., 2003), suggesting a restricted host range. Further indications of the host specific nature of *V. invictae* is its lack of detection in the fire ant parasitoid fly, *Pseudacteon obtusus* Borgmeier, while *K. solenopsae* has been detected in *P. obtusus* and other species of the *Pseudacteon* flies (Oi et al., 2009).

Over 20 ant species have been reported to co-occur with *S. invicta* in North America (King and Tschinkel, 2006). Given the current inability to directly transmit *V. invictae* with isolated spores, it was beyond the scope of this study to examine other co-occurring ant species of North America. The results of the laboratory challenge tests reported here are supported by field studies of *V. invictae* in South America. *V. invictae* was not found in 12 genera and 19 species (509 and 438 individuals near Corrientes and San Javier, Argentina, respectively) of sympatric non-*Solenopsis* ants, nor in 43 families and 80 species (235 individuals) of non-ant arthropods (Porter et al., 2007). Similarly, Briano et al. (2002) reported infections in 3 of 4 of *Solenopsis* species collected, but none in nine genera of ants. The two native fire ants tested in our study are in the *Solenopsis geminata* species group with a distribution (Tschinkel, 2006) that is not sympatric with the known distribution of *V. invictae* (Briano et al., 2006; Jouvenaz and Ellis, 1986; Jouvenaz et al., 1980). The host range of *V. invictae* in the field and the finding that *S. geminata*, *S. xyloni*, and *L. humile* are refractory to laboratory inoculations indicate that *V. invictae* is specific to imported *Solenopsis* ants in the *Saevissima* group, and provide strong evidence that the release of *V. invictae* in the US as a biological control agent poses little or no risk to US native ants and other arthropods.

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