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Journal of Invertebrate Pathology 88 (2005) 108–115
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Transmission of *Vairimorpha invictae* (Microsporidia: Burenellidae) infections between red imported fire ant (Hymenoptera: Formicidae) colonies

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Received 1 October 2004; accepted 19 November 2004
Available online 20 January 2005

Abstract

Red imported fire ant, *Solenopsis invicta*, colonies were successfully infected with the microsporidium *Vairimorpha invictae* by introducing live larvae, pupae, or dead adults from *V. invictae*-infected field colonies collected in Argentina. Introductions with 4th instar larvae or non-melanized pupae obtained from infected field colonies, resulted in infection of 40% of the inoculated colonies. Introductions of 4th instars or melanized pupae produced from colonies that were initially infected in the laboratory, resulted in infections of 83% of the colonies, thus perpetuating the infection in other colonies. Infection was detected in 2 of 6 colonies after introducing adult worker caste ants that had died with *V. invictae*. The average number of adults and the volume of immature ants per colony were significantly lower in the infected than in the control colonies. Infected colonies had 86% fewer adults per colony and 82% less immature ants than the controls. A portion of the 16S rRNA gene of the *V. invictae* identified from these studies was amplified, cloned, and sequenced; the 1251 nucleotide amplicon was 100% identical to the 16S rRNA gene sequence recorded previously in the GenBank database, thus verifying the species as *V. invictae*. This is the first report of the artificial transmission of this pathogen to uninfected ant colonies, and demonstration of its ability to hinder growth in individual colonies.
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Keywords: *Vairimorpha invictae*; Microsporidia; *Solenopsis invicta*; Fire ant; Pathogen; Transmission; Biological control

1. Introduction

Vairimorpha invictae is a microsporidium that was first described by Jouvenaz and Ellis (1986) infecting a colony of red imported fire ants, *Solenopsis invicta*, collected in the state of Matto Grosso, Brazil. *V. invictae* produces dimorphic spores, where large numbers of binucleate free spores occur in pupae and adult ants, and uninucleate octospores contained in sporophorous vesicles are present only in adults (Jouvenaz and Ellis, 1986). This contrasts with *Thelohania solenopsae* another microsporidium that infects fire ants, which has four reported spore types that are present in various ant developmental stages. Specifically, uninucleate octospores, or meiospores, contained in sporophorous vesicles are present in adults and pupae (Knell et al., 1977); binucleate, *Nosema*-like spores, or free spores, present primarily in adults but also reported in pupae and larvae (Knell et al., 1977; Sokolova et al., 2004); binucleate spores from pupae (Shapiro et al., 2003); and binucleate megaspores observed in adults and brood (Sokolova et al., 2004). A reported fifth spore, the macrospore, was recently considered to be abnormal octospores, or teratospores (Sokolova et al., 2004).

Surveys for pathogens of fire ants in South America indicated that *V. invictae* had a moderately low overall

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0022-2011/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.jip.2004.11.006

prevalence, being found in 2.3% of 2528 colonies surveyed (Briano and Williams, 2002). However, at several *S. invicta* sites in Santa Fe Province, Argentina, *V. invictae* reached epizootic levels with 50% of the colonies infected (Briano and Williams, 2002). In a survey of black imported fire ants, *Solenopsis richteri*, and *Solenopsis quinquecuspis* in Buenos Aires Province, Argentina, *V. invictae* was detected in 1% of 1836 colonies compared to 8% for *T. solenopsae* (Briano et al., 1995). Preliminary observations of natural field infections of *V. invictae* in *S. invicta* suggested that this pathogen could reduce colony populations and had the potential to be an effective biological control agent (Briano et al., 2002). In addition, enhanced decline in colonies has been suggested in natural simultaneous infections of *V. invictae* and *T. solenopsae* (Briano, 2005). To further assess the potential of *V. invictae* as a biological control agent for introduction into the US, infections initiated in the laboratory would facilitate host specificity and colony impact testing. Previous attempts to transmit this pathogen to small (10,000 adult ants), uninfected *S. invicta* colonies by providing boiled chicken egg yolk wetted with spore suspensions or by introducing brood from infected colonies were unsuccessful (Jouvenaz and Ellis, 1986). Nevertheless, our objectives were to infect *S. invicta* colonies with *V. invictae* under laboratory conditions and to document the impact of infection on colony growth.

2. Materials and methods

Vairimorpha invictae-infected *S. invicta* colonies were collected near San Javier, Santa Fe province, Argentina in 2003 and transported to quarantine facilities at the USDA-ARS Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, Florida. Infected colonies were identified by examining aqueous extracts of macerated groups of adults for spores of *V. invictae* and *T. solenopsae* using phase contrast microscopy (Briano and Williams, 2002) and comparing morphological features of free spores and octospores (meiospores) described by Jouvenaz and Ellis (1986). Voucher specimens of *V. invictae* were deposited in the collection of the senior author in the form of giemsa-stained slides and alcohol preserved ants from infected colonies. Additional slides were added to the collection of Dr. James Becnel at the USDA-ARS Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, Florida, USA.

2.1. Molecular and morphological verification of microsporidian species

To further verify the identity of *V. invictae*, the 16S rRNA gene was amplified, cloned, sequenced, and compared with sequences in the GenBank database. DNA was extracted from *V. invictae*-infected *S. invicta* by the method of Valles et al. (2002). The *S. invicta* was obtained from a colony that was infected in the study described in Sections 2.3 and 3.3. Oligonucleotide primers were designed to the *Vairimorpha* sp. 16S rRNA gene sequence (Accession No. AF031539) reported previously by Moser et al. (1998). PCR was carried out with forward (5′-TCAGAGATTAAGCCATGCAAGCCAG) and reverse (5′-TGTATCCAATCTACAAGCACA GATTCGTC) oligonucleotide primers under the following temperature regime: 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 57 °C for 15 s, and 68 °C for 2 min, followed by a polishing step of 68 °C for 5 min. The 1251 nucleotide amplicon was ligated into the pCR-4 vector and transformed into TOP TEN competent *Escherichia coli* (In Vitrogen, Carlsbad, CA). Four clones were randomly selected and sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida. Contiguous nucleotide sequences were analyzed by BLAST (Altschul et al., 1997) and sequence comparisons were made using the Vector NTI Suite ver. 7.0 (Informax, Bethesda, MD).

Wet mounts were prepared from groups of approximately 10 macerated adult workers from each of two colonies that were inoculated with *V. invictae* infected brood (Sections 2.2 and 2.3). Worker samples were collected at least 28 weeks after inoculation. The length and width of free spores and meiospores were measured with an image-splitting micrometer (Vickers A.E.I., Vickers Instruments, Malden, MA) (Undeen, 1997).

2.2. Inoculations with larvae or non-melanized pupae from field colonies

Inocula consisted of brood collected from a colony infected with *V. invictae*. Infection rates of the inocula were determined to be 100% based on both individual wet mounts of 10 pupae and 10 giemsa-stained larval smears (Undeen, 1997) that were examined by microscopy for *V. invictae* spores or vegetative stages, respectively. Incipient colonies used for inoculations and controls were reared (Banks et al., 1981; Oi and Williams, 2003) from *S. invicta* queens, that were collected within a day of nuptial flights (newly mated queens), in Alachua and Orange counties Florida. Colonies most likely possessed the monogyne genotype, because queens with this genotype have the most capacity to disperse and independently found colonies (DeHeer et al., 1999). Colonies were inoculated when they were 5–6 weeks old and contained approximately 400 pieces of brood (1 ml), 30 workers, and 1 queen. Because removing ants from such small incipient colonies could impair the colonies, and because *V. invictae* is not known to occur in the US (Beckham et al., 1982; Broome, 1974; Jouvenaz, 1986; Jouvenaz et al., 1977; Pereira et al., 2002; D.H.O. unpublished data), colonies were not examined for infection

prior to inoculation. To support the assumption that colonies were not previously infected, wet mounts of 3–5 groups of approximately 50 adult workers per control colony were collected between 14 and 28 weeks after inoculation and examined for *V. invictae*. Inocula consisted of either 10 live, 4th instar larvae or 10 live, non-melanized pupae. The larvae and pupae were placed at the entrance of nesting tubes and adult workers would carry them into the colony. Larval and pupal inocula were introduced into five colonies each and brood was not introduced into any of the control colonies ($N=5$).

Vairimorpha invictae infection was determined by examining wet mounts of 24–48 individual pupae per colony that were collected 8, 9, 12, and 28 weeks after inoculation. In addition, 17 weeks after inoculation, an average of 32 (range 6–40) melanized pupae were isolated per colony ($N=12$, remaining 3 colonies had no pupae) to allow adult workers to emerge. For each colony, groups of these adults (ca. 10 per group) were then macerated and the aqueous extract examined for spores. In addition, all samples were examined for evidence of *T. solenopsae* infections.

The volume of brood and the number of adults were visually estimated biweekly for 26 weeks (except for readings taken at 9 instead of 10 weeks and no reading at 22 weeks) by visual comparison with known volumes of brood and counts of live ants. The presence of a live queen was also recorded. Brood volume and the number of adults were averaged over samplings from weeks 9 through 26, which was when changes in these parameters were manifested, and compared by one-way analyses of variance and Tukey's HSD test (SAS Institute, 2001), among infected, inoculated uninfected, and control colonies. Analysis of adult populations was conducted on transformed data [$\log_{10}(x+1)$] to reduce heterogeneity among variances.

2.3. Inoculations with larvae and melanized pupae obtained from laboratory infected colonies

The source of the inocula for this study was a colony that was infected in the laboratory from the first pupae inoculation described previously. The infection rate of a subsample of the pupae was 100% ($N=10$). Groups of 10 melanized pupae from the *V. invictae*-infected colony were each added to three, incipient *S. invicta* colonies, each containing about 30 workers, about 200 pieces of brood (0.5 ml), and 1 queen. Similarly, groups of 10 4th instar larvae from the same colony were added to another set of three incipient colonies. Three additional incipient colonies were not inoculated and used as controls. Incipient colonies were reared from newly mated queens collected in Florida and were not examined for infection before inoculation for reasons described previously. To support the assumption that colonies were not infected prior to inoculation, wet mounts of groups of adult workers (~50 workers/group) from each control colony were examined for *V. invictae* spores. To determine if colonies became infected, individual non-melanized pupae ($N=10$ per sample) were examined for *V. invictae* spores from each colony at 8, 12, and 16 weeks after inoculation. In addition, for the melanized pupae introductions, 15 non-melanized pupae per colony were examined 6 weeks after inoculation. Giemsa-stained 4th instar larvae ($N=10$ per colony) also were examined for vegetative stages of *V. invictae* at 8 weeks. The social form, monogyny or polygyny, was determined for infected colonies using the procedure of Valles and Porter (2003).

Brood volume and the number of adults from infected and control colonies were averaged over samplings from weeks 8 through 16. Averages per colony were compared by one-way analyses of variance (SAS Institute, 2001), with the analysis on adult populations being conducted on transformed data [$\log_{10}(x+1)$] to stabilize variances.

2.4. Inoculations with dead adults

Dead adult workers, obtained from the midden piles of six, *V. invicta*-infected colonies collected from Argentina, were used to inoculate six incipient colonies containing about 30 workers, 1 ml brood, and 1 queen. The infection rate of one sub-sample of dead ants used as inocula was estimated to be about 30%. This was determined by examining wet mounts of individual dead ants ($N=10$) for either type of *V. invictae* spores. Because dead workers collected from colony middens were usually desiccated, examination of individual ants for spores was difficult. Thus, only the presence of *V. invictae* spores was confirmed in the aqueous extracts of groups of macerated dead workers for the other five colonies. To inoculate colonies, 0.2 g (ca. 300 ants) dead workers were placed near the entrance of nesting tubes. Control colonies ($N=6$) did not receive any dead ants. Infection was determined by examining individual pupae and/or groups of live workers for spores as described previously, generally at 8, 17, and 33 weeks after inoculation. Due to the decline of some of the colonies, infection examinations also occurred at 3, 10, 16, 21, and 25 weeks after inoculation. Colonies were not examined for infection before inoculation for reasons indicated previously. Brood volume, the number of live adults, and queen survivorship were monitored biweekly for 14 weeks and then at 16 or 17, and 19 weeks. Colonies were maintained up to 33 weeks after inoculation in order to determine if *V. invictae* infection would develop after an extended period of time.

Averages of brood volume and of number of adults per infected colony, per inoculated but not infected colony, and per control colony over weeks 8–17 were compared by one-way analyses of variance and Tukeys HSD test (SAS Institute, 2001). Analyses on brood volume

and adult populations were conducted on transformed data [$\log_{10}(x+1)$] to reduce heterogeneity of variances.

3. Results

3.1. Molecular and morphological verification of microsporidian species

The 1251 nucleotide amplicon amplified from *V. invictae*-infected *S. invicta* was 100% identical to the 16S rRNA gene sequence reported previously by Moser et al. (1998). Thus, the species Moser et al. (1998) had identified from *S. richteri* and the species we collected years later from *S. invicta* are most likely the same species, namely *V. invictae*. Microscopic observations revealed binucleate, bacilliform, free spores and uninucleate, ovoid, and meiospores consistent with previous descriptions by Jouvenaz and Ellis (1986) of *V. invictae* from *S. invicta* and Moser (1995) of *Vairimorpha* sp. from *S. richteri*. Free spore and meiospore dimensions averaged over both colony samples were $3.03 \pm 0.14 \times 10.34 \pm 0.56\,\mu m$ (±SD, $N = 12$) and $4.02 \pm 0.37 \times 6.25 \pm 0.17\,\mu m$ ($N = 12$), respectively. These dimensions were within the range of *V. invictae* free spore and meiospore widths and lengths reported by Jouvenaz and Ellis (1986) of $3.1 \pm 0.3 \times 11.2 \pm 3.4\,\mu m$ and $4.2 \pm 0.7 \times 6.3 \pm 0.2\,\mu m$, respectively. Similarly, our range of spore dimensions overlapped the dimensions reported by Moser (1995), where free spores were $3.11 \pm 0.19 \times 10.76 \pm 0.48\,\mu m$, and meiospores were $4.31 \pm 0.25 \times 6.45 \pm 0.61\,\mu m$.

3.2. Inoculations with larvae or non-melanized pupae from field colonies

From the inoculations with 4th instar larvae, *V. invictae* infection was obtained in 2 of 5 colonies. Similarly, infection was found in 2 of 5 colonies inoculated with non-melanized pupae. Free spores were detected in 19 of 24 and 33 of 42 pupae sampled from the two, larvae-inoculated colonies. From infected colonies inoculated with pupae, 43 of 45 and 41 of 46 pupae contained free spores. Hence, more infected pupae were recovered than the number of larvae or pupae introduced as inocula, indicating that *V. invictae* had spread in those colonies (Table 1). Furthermore, infected pupae were collected at 8, 9, 12, and 28 weeks after inoculation which is well beyond the 11.1–12.4 days reported for *S. invicta* development from 4th instars to adult workers at 28–30 °C (Porter, 1988), thus it is unlikely that we were sampling inocula. Free spores and meiospores were observed in adult worker groups that enclosed from isolated pupae obtained from two infected colonies inoculated with either developmental stage. The other two infected colonies could not be sampled due to a lack of pupae because of colony decline. *V. invictae* spores were not observed in the remaining samples of workers from the uninfected inoculated colonies and the controls. *T. solenopsae* spores were not observed in any samples, and all control colonies were uninfected.

Colony growth in infected colonies was significantly slower than in the control colonies as measured by brood volume ($F = 4.22$; df = 2, 12; $P = 0.041$) and number of workers ($F = 4.08$; df = 2, 12; $P = 0.045$) from weeks 9 to 26. For colonies that were inoculated, but infections were either not detected, or detected in less pupae than the number of larvae or pupae used as inocula, colony growth was intermediate between the infected and control colonies (Fig. 1 and Table 2). One of the queens in the four infected colonies died (at 6–8 weeks) before termination of the study at 26 weeks. Infection in the remaining queens was not determined because they could not be sacrificed for examinations as they were needed for future studies.

Table 1
Source, type, and amount of inocula (immature or adult ants collected from *V. invictae*-infected colonies) added to individual, uninfected *S. invicta* colonies and the number of individual pupae or larvae infected with *V. invictae*

Inocula source[a]	Inocula type (No. added per colony)	% colonies infected (*N*)	Min.–Max. No. pupae infected/No. sampled per colony	Min.–Max. No. larvae infected/No. sampled per colony	No. adult groups[c] Infected/No. sampled among colonies
Field	4th instars (10)	40 (5)	19/24–33/42	—[b]	0/0[d], 3/3, 0/4, 0/4, 0/5
Field	Pupae (10)	40 (5)	41/46–43/45	—	1/1, 4/4, 0/4, 0/4, 0/0[d]
Control	None	0 (5)	0/22–0/35	—	0/3, 0/4, 0/5, 0/5, 0/5
Lab	4th instars (10)	67 (3)	24/30–28/30	3/10–4/10	—
Lab	Melanized pupae (10)	100 (3)	17/45–40/45	6/10–9/10	—
Control	None	0 (3)	0/20	—	—
Field	Dead adults (≈300)	33 (6)	2/20–6/10	—	0/1, 1/1
Control	None	0 (6)	0/30	—	0/1, 0/1, 0/1, 0/1, 0/1, 0/1

Column 6 provides the number of *V. invictae*-infected groups of adult workers examined per colony. All samples were obtained at least 8 weeks after inoculations.

[a] *V. invictae* inocula was obtained from field-collected *S. invicta* colonies or laboratory-infected and reared *S. invicta* colonies.

[b] —, No samples examined.

[c] Approximately 50 workers per group.

[d] Worker groups not examined.

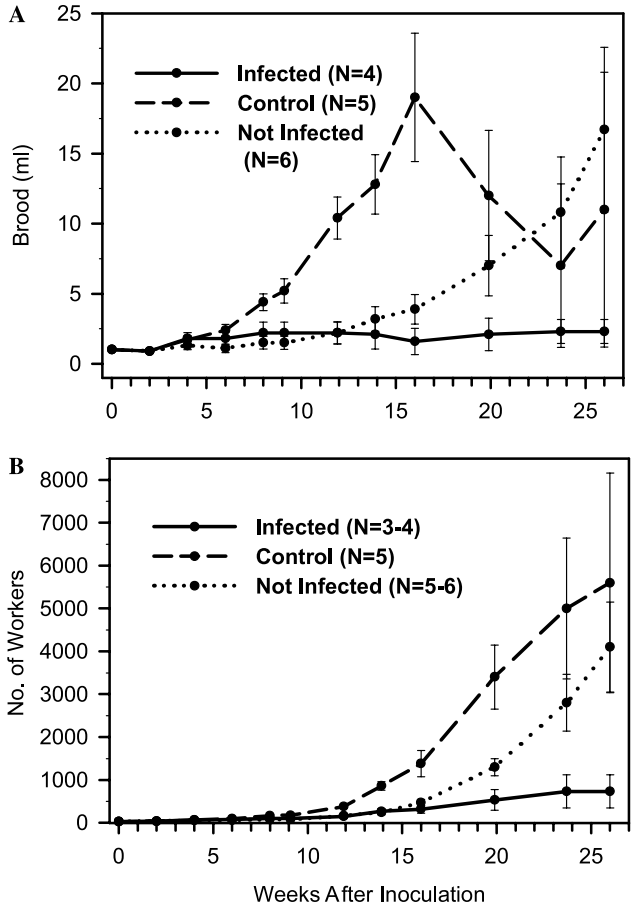

Fig. 1. Mean (±SEM) brood volume (A) and adult workers (B) per colony from *V. invictae* infected, inoculated but not infected, and control *S. invicta* colonies after inoculations with brood from *V. invictae* infected, *S. invicta* colonies collected from the field in Argentina.

3.3. Inoculations with larvae and melanized pupae obtained from laboratory-infected colonies

Infections of *V. invictae* were observed in all three of the colonies inoculated with melanized pupae and in 2 of 3 colonies inoculated with larvae (Table 1). Infection was not detected in any of the control colonies. Infected colonies possessed the monogyne genotype ($Gp\text{-}9^{BB}$). Brood volume ($F = 21.63$; df = 1, 6; $P < 0.004$) and adult worker population ($F = 44.47$; df = 1, 6; $P < 0.001$) per colony in the infected colonies were significantly lower than the control colonies. These reductions occurred when samples were obtained from 8 to 16 weeks after inoculation. The single inoculated colony that did not become infected was excluded from the analysis. At the end of the study, 16 weeks after inoculation, growth in brood and worker levels were reduced 3- and 14.6-fold, respectively, in the infected colonies relative to the controls (Fig. 2). All of the queens from the five infected colonies were still alive through the end of the study. Infection in these queens could not be determined because they were needed for further studies and thus could not be sacrificed.

3.4. Inoculations with dead adults

Vairimorpha invictae was detected in 2 of 6 inoculated colonies. In one colony, infections were found in 6 of 10 pupae and a group of adult workers. Infection in the queen was unknown as she probably died and was dismembered, and thus could not be recovered. The other colony had only a 10% ($N = 20$ pupae) infection rate at 8 and 10 weeks after infection, and infection was not detected in the queen that died. However, both colonies had significant reductions in brood ($F = 11.61$; df = 2, 8; $P = 0.004$) and adult worker populations ($F = 15.91$; df = 2, 8; $P = 0.002$) between the infected and control colonies (Table 2, Fig. 3), and premature death of queens at 14 and 16 weeks after inoculation. By 16 and 17 weeks after inoculation, brood was absent in both colonies, while the average brood level per colony in the controls increased 22-fold. Adult populations in the infected colonies were 15 times lower than the control colonies at 16/17 weeks.

Table 2
Mean brood volume and mean number of workers from *V. invictae* infected, inoculated but not infected, and control *S. invicta* colonies for inoculations with infected brood from field-collected colonies (Study 1), brood from laboratory-infected colonies (Study 2), and dead workers (Study 3)

Infection status	Study 1[a]: Mean population per colony over weeks 9–26		Study 2[b]: Mean population per colony over weeks 8–16		Study 3[c]: Mean population per colony over weeks 8–17	
	Brood (ml)	No. workers[d]	Brood (ml)	No. workers[d]	Brood (ml)[d]	No. workers[d]
Control	11.1a[e]	2399.7a	21.8a	2333.3a	6.5a	512.5a
Not infected[f]	6.5ab	1104.9ab	19.2-[g]	670.0-[g]	4.8a	299.2a
Infected	2.1b	321.8b	6.4b	332.0b	0.3b	71.3b

[a] Colonies inoculated with larvae or non-melanized pupae. Control ($N = 5$); not infected ($N = 6$); infected ($N = 4$).
[b] Colonies inoculated with larvae or melanized pupae. Control ($N = 3$); Not infected ($N = 1$); infected ($N = 5$).
[c] Colonies inoculated with dead adults. Control ($N = 6$); not infected ($N = 3$); infected ($N = 2$).
[d] Analysis conducted on $\log_{10}(x + 1)$ transformed data, untransformed means presented.
[e] Means within a column followed by the same letter are not significantly different ($P > 0.05$) by analysis of variance and Tukey's HSD test.
[f] Inoculated but infection not detected.
[g] Excluded from analysis due to insufficient replication ($N = 1$).

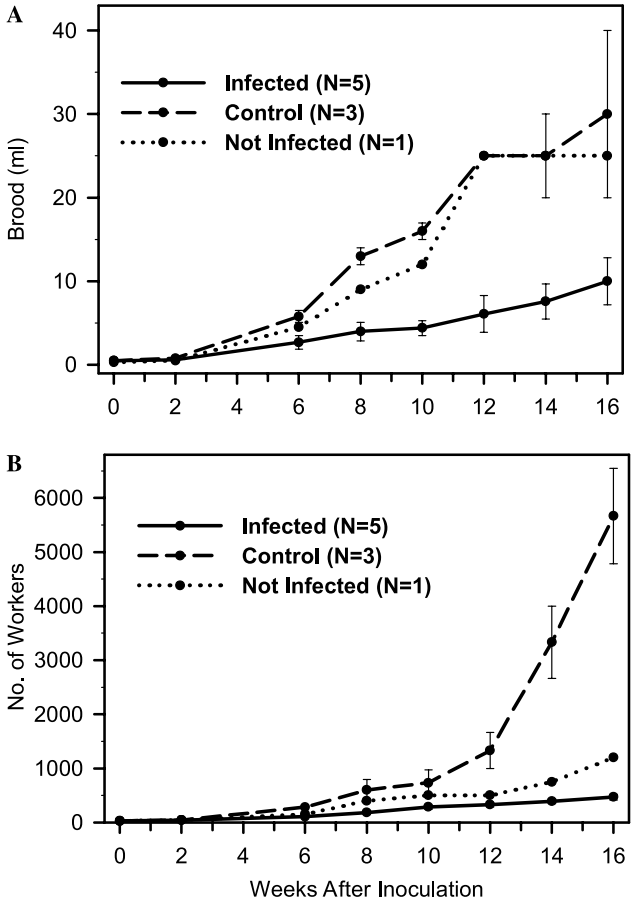

Fig. 2. Mean (±SEM) brood volume (A) and adult workers (B) per colony from *V. invictae* infected, inoculated but not infected, and control *S. invicta* colonies after inoculations with brood from *S. invicta* colonies infected with *V. invictae* in the laboratory.

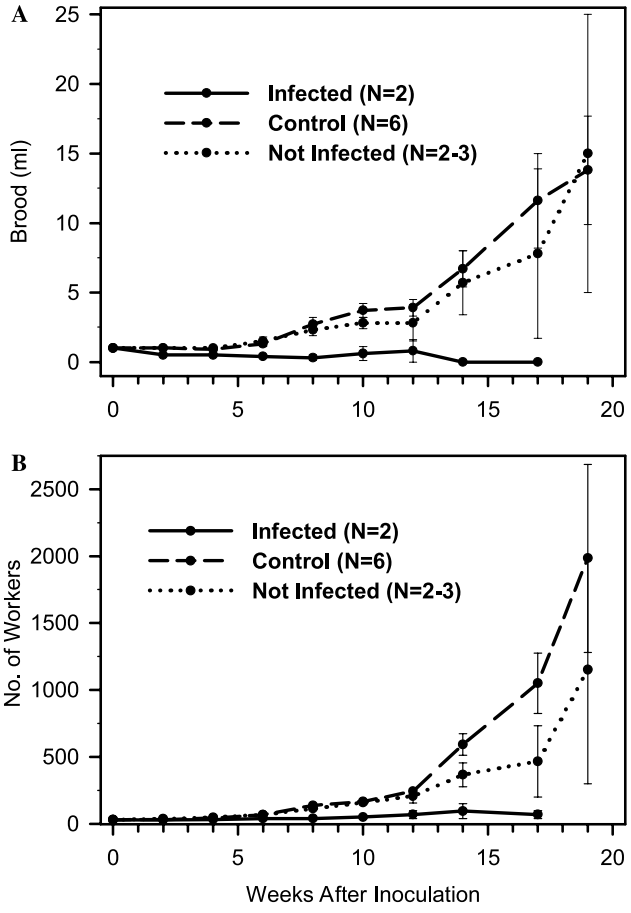

Fig. 3. Mean (±SEM) brood volume (A) and adult workers (B) per colony from *V. invictae* infected, inoculated but not infected, and control *S. invicta* colonies after inoculations with dead adults collected from *V. invictae* infected, *S. invicta* colonies obtained from the field in Argentina.

4. Discussion

This is the first report of the laboratory transmission of *V. invictae* to uninfected ant colonies. We were able to successfully transmit *V. invictae* from field collected *S. invicta* colonies to laboratory *S. invicta* colonies, and subsequently perpetuate the infection in other previously uninfected laboratory colonies using inocula produced by the initial laboratory-infected colony. The similar pattern of suppressed colony growth in *S. invicta* colonies infected by brood from either the field, or from the laboratory-infected colonies, indicated that the laboratory reared inocula maintained its pathogenicity. Inoculated colonies that did not become infected exhibited a temporary suppression in growth. This possibly could have been the result of an unapparent initial infection that was not sustained, thus allowing for colonies to recover. While uninfected brood was not added to control colonies, the addition of brood to incipient colonies generally facilitates fire ant colony establishment and growth (Tschinkel, 1992). Hence, the addition of brood most likely did not the cause the delay in colony growth.

Vairimorpha invictae infections were obtained using 4th instars, non-melanized pupae, and melanized pupae. Due to the limited number of inoculations, rates of colony infection among these inocula could not be compared statistically but larval and pupal inoculations resulted in relatively similar colony infection rates of 50 and 63%, respectively. Infecting fire ant colonies with *V. invictae* by introducing infected larvae and pupae was the same method used to transmit *T. solenopsae* (Williams et al., 1999). However, Oi et al. (2001) had a higher rate of transmission using infected 4th instar larvae when compared to infected pupae as inocula (80 and 37.5%, respectively).

Queens were still alive when studies were terminated after 16 or 26 weeks in 8 of 9 infected colonies inoculated with brood. In contrast, premature queen death was reported by Williams et al. (1999) in 80% of older and larger laboratory *S. invicta* colonies infected with *T. solenopsae.* However, rates of queen mortality and colony decline are not directly comparable between the *V. invictae* and *T. solenopsae* infections in these studies because the inoculated colonies differed in age.

This study used incipient colonies where queens are very young, and still may be utilizing their own energy reserves to maintain survivorship (Vinson and Greenberg, 1986). The study by Williams et al. (1999) used well-established colonies produced by older queens that rely solely on the colony for their nutrition, which may facilitate their infection. Oi and Williams (2003) reported that a majority of *T. solenopsae*-infected *S. invicta* queens, which were collected in the field soon after nuptial flights and established colonies, died prematurely (within 26 weeks). We did not detect *V. invictae* infection in the single queen from an infected colony that died in our studies. Infection in the other live queens from infected colonies was not determined because they were needed for other studies and thus were not macerated and examined for spores. Less destructive attempts to find evidence of queen infection, such examining eggs and brood reared from isolated queens via PCR (Valles et al., 2004) from infected colonies yielded very small sample sizes and inconclusive results. Briano and Williams (2002) reported a low prevalence of infection in mated queens and eggs and were uncertain of the significance of transovarial transmission in the life cycle of this pathogen. This is in contrast to *T. solenopsae*, where queen and egg infection has been consistently documented (Briano et al., 1996; Knell et al., 1977; Oi and Williams, 2002, 2003; Valles et al., 2002).

We also achieved transmission of *V. invictae* by the addition of infected dead adults into colonies. Queen death occurred within 16 weeks of inoculation in the two colonies that *V. invictae* was detected. While the number of colonies with *V. invictae* was limited in this study, a preliminary study also resulted in infection in 1 of 5 colonies inoculated with dead, *S. invicta* workers collected from *V. invictae*-infected colonies from Argentina (D. H. O. unpublished data). This infected colony also had a similar pattern of decline with the queen dead at 16 weeks after inoculation and no brood remaining. While queen death occurred more frequently in the successful dead-worker inoculations than with the brood inoculations, the association of inocula type and queen mortality needs further confirmation.

Infection with dead workers and the inability to confirm infection in queens suggested that infection may be initiated directly into larvae. *S. invicta* colonies use their 4th instar larvae to digest solid food which is then passed by trophallaxis to the rest of the colony (Petralia and Vinson, 1978; Vinson, 1997). Digesting or liquefying solids is imperative to obtaining nutrition from solid foods because adult *S. invicta* workers possess a filtering buccal tube that prevents ingestion of particles >1 μm. In contrast 4th instars, have been shown to ingest particle sizes >45 μm (Glancey et al., 1981). Presumably worker ants can feed spore laden cadavers to the larvae possibly resulting in the ingestion of *V. invictae* spores which are >3 μm (Jouvenaz and Ellis, 1986). Direct infection of larvae occurs with the microsporidian pathogen *Burnella dimorpha* in the tropical fire ant, *Solenopsis geminata*, where worker ants mechanically transfer spores from pupae to larvae (Jouvenaz et al., 1981). There also is indirect evidence of direct infection of larvae by infected workers with *T. solenopsae* in *S. invicta* (Allen and Knell, 1980; Oi et al., 2001). Prevalence of *V. invictae* in inocula added to colonies that became infected could not be determined due to desiccation of individual dead workers collected from colony middens. Briano and Williams (2002) reported a greater prevalence of infection in dead workers than in live workers from *V. invictae*-infected colonies. The potential to use dead adults as inocula, may make the logistics of establishing this pathogen as a biological control agent more feasible, perhaps by incorporating spores into an ant bait.

The ability to transmit *V. invictae* to uninfected *S. invicta* colonies allowed for the documentation of the negative impact this pathogen can have on individual colonies. Infected colonies had 86% fewer adults per colony and 82% less immature ants than the controls. The observations of dramatic declines in field populations of *S. invicta* when simultaneous infections of *T. solenopsae* and *V. invictae* occur (Briano et al., 2002) can now be tested in individual colonies as both pathogens can be artificially transmitted to uninfected colonies. With the geographic distribution of natural infections of *T. solenopsae* increasing in US imported fire ant populations (Pereira, 2003; Williams et al., 2003), the ability to efficiently initiate field infections of *V. invictae* could result in faster declines in fire ant populations where such dual infections are established.

Acknowledgments

Technical assistance provided by, Eileen Carroll, Christine Harrison, Euripides Mena, and Chuck Strong (all currently or formerly of the USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology) was greatly appreciated. Reviews of an earlier draft of the manuscript were graciously provided by Faith Oi (University of Florida) and James Becnel (USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology).

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