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Pseudacteon decapitating flies (Diptera: Phoridae): Are they potential vectors of the fire ant pathogens *Kneallhazia* (=*Thelohania*) *solenopsae* (Microsporidia: Thelohaniidae) and *Vairimorpha invictae* (Microsporidia: Burenellidae)?

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ABSTRACT

Fire ant decapitating flies in the genus *Pseudacteon* were tested for their potential as hosts or vectors of two microsporidian pathogens of the red imported fire ant, *Solenopsis invicta*. Decapitating flies that attacked or were reared from *S. invicta* workers infected by *Kneallhazia* (=*Thelohania*) *solenopsae* or *Vairimorpha invictae* were tested for either pathogen by polymerase chain reaction (PCR) tests or visual examination for spores using phase microscopy. Three species of fire ant decapitating flies acquired the pathogen, *Kneallhazia solenopsae*. *K. solenopsae* was detected in 58% of pooled samples of *Pseudacteon obtusus* flies and 44% of pooled samples of *Pseudacteon cultellatus* that developed in *K. solenopsae*-infected fire ant workers. *K. solenopsae* was also found in 17% of pooled samples of field-collected *Pseudacteon curvatus*. In contrast, the microsporidium *V. invictae* was not detected in *P. obtusus* reared from *V. invictae*-infected *S. invicta workers*. Neither *K. solenopsae* nor *V. invictae* were detected in any of the hovering or ovipositing flies in the laboratory exposures, indicating no mechanical acquisition of the microsporidia occurred during oviposition activity. Greater than 92% of the *P. obtusus* that developed in *K. solenopsae*-infected ants survived and emerged as adults, thus indicating no detrimental effects of the microsporidium on pupal development and emergence. These results indicate that *Pseudacteon* decapitating flies may be able to vector *K. solenopsae* but not *V. invictae* among fire ants. Further tests are planned to determine if flies containing *K. solenopsae* are capable of transferring this pathogen either during oviposition or by being consumed by fire ant larvae.

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1. Introduction

Natural enemies of the red imported fire ant, *Solenopsis invicta* Buren, from South America have been released and/or dispersed in the United States of America (USA) since 1997 (Williams et al., 2003). The microsporidian pathogens of fire ants *Kneallhazia solenopsae* Knell, Allen and Hazard [recently removed from the genus *Thelohania* (Sokolova and Fuxa, 2008)] and *Vairimorpha invictae* Jouvenaz and Ellis alone and in combination are associated with localized declines of 53–100% in *S. invicta* populations in Argentina (Briano, 2005). Infected laboratory colonies of either pathogen exhibit lower brood production and debilitation of infected queens (Williams et al., 1999; Oi et al., 2005). *K. solenopsae* is present in the USA while *V. invictae* is under evaluation for release (Oi and Valles, 2008). Parasitic flies in the genus *Pseudacteon* have been released for the biological control of fire ants in the USA. Four species (*Pseudacteon tricuspis* Borgmeier, *Pseudacteon curvatus* Borgmeier, *Pseudacteon litoralis* Borgmeier and *Pseudacteon obtusus* Borgmeier) have been established (Graham et al., 2003; Porter et al., 2004; Vazquez et al., 2006; Gilbert et al., 2008; Graham, personal communication). These flies are endoparasitoids; an egg oviposited into an adult fire ant worker hatches into a maggot that migrates to the head of the ant where it develops and pupates. The parasitized ant head usually detaches from the ant body prior to fly pupariation (Porter, 1998).

Microsporidian infections can occur in parasitoid wasps and flies that develop in infected hosts (Becnel and Andreadis, 1999). Deleterious effects on infected wasps may impact integrated pest management programs where parasitoids are an important component (Schuld et al., 1999) or change the species prevalence within a complex of natural enemies (Cossentine and Lewis, 1988). Establishment of the queen debilitating microsporidian pathogens and the fire ant decapitating flies were thought to be complimentary biological control agents because they affect different castes within fire ant colonies. However, whether the endoparasitic flies could successfully develop in microsporidia-infected adult worker caste fire ants had not been examined. The objectives of this study were to (1) determine if fire ant decapitating *Pseudacteon* phorid flies could develop to adulthood in fire ants infected with *K. sole-*

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nopsae or *V. invictae* and (2) to determine if either of the microsporidia could be found in adult flies that emerged from infected hosts. The presence of fire ant pathogens in adult flies would be particularly interesting because it opens up the possibility that these flies may vector certain fire ant diseases (Valles and Porter, 2007).

2. Materials and methods

2.1. Vairimorpha invictae exposure

Vairimorpha invictae-infected *S. invicta* colonies were collected in San Javier, Santa Fe province, Argentina and shipped to the quarantine facility at the USDA-ARS Center for Medical, Agricultural and Veterinary Entomology in Gainesville, Florida in 2007. Adult worker ants from two colonies were sedated with ether and sieved through a #12 mesh to obtain large-sized ants which favor the development of *P. obtusus* flies. Infection rates of the sieved workers were 85% for each colony based on individual wet mounts of the workers (n = 20 per colony) examined for spores by phase-contrast microscopy at 400×. Ants were divided into four, 1-g groups per colony (ca. 300–400 ants/group) and each held overnight with 1 g of brood separated from field-collected *S. invicta* colonies to facilitate brood adoption. Ants and brood were exposed for 3 days to *P. obtusus* in an attack box (see Vogt et al., 2003), where adult ants carry brood between harborages that constantly switch from being inaccessible (suspended above the tray) to accessible (on the tray floor). The transfer of brood between harborages overcomes the adult workers' normal behavior of hiding from ovipositing flies (Vogt et al., 2003).

After 3 days of exposure to flies, adult ants were separated from the brood and held in rearing containers until ant decapitation (10–25 days after initial exposure to flies). Ant heads and bodies were collected individually and held separately according to the following categories: (1) heads that could be matched definitively with bodies (head partially attached to body); (2) heads that probably belonged to particular bodies (head near body); (3) heads that could not be assigned to specific bodies. Ant bodies that could be matched with heads (categories 1 and 2) were individually examined for *V. invictae* spores by microscopy, thus allowing for the determination of whether flies developed in infected ants. A subsample of bodies from category 3, also were examined individually for spores by microscopy to estimate infection rates of the parasitized ants. Adult *P. obtusus* that emerged from the first two groups were separated by sex and grouped into samples of 7–13 individuals and tested for the presence of *V. invictae* by polymerase chain reaction (PCR). *P. obtusus* adults that emerged from unassigned heads were sexed and grouped into sets of 16–21 flies for *V. invictae* detection.

Female *P. obtusus* flies that were hovering within the trays in oviposition mode were aspirated and pooled into sets of 14 or 15. Flies were examined for the presence of *V. invictae* by PCR to determine if there was evidence of ovipositing flies acquiring *V. invictae* and thus potentially being mechanical vectors. Several groups of males were also tested in the event that venereal transmission occurred.

2.2. Kneallhazia solenopsae exposure

Three *K. solenopsae*-infected *S. invicta* colonies were collected from Alachua County, Florida and maintained in the laboratory. Because a sufficient number of large ants were difficult to obtain, large and the bigger media-sized worker ants were separated from the colonies by sieving, as described previously but with a #14 mesh screen, and by segregating ants with forceps. The average infection rate of the isolated workers per colony was 72% (range 65–80%, n = 20 ants/colony). For each colony, separated ants were divided into two, 1-g groups (ca. 700 ants/group) and each held overnight with 1.5 g of brood separated from field-collected *S. invicta* colonies. Ants and brood were exposed for 3 days to *P. obtusus* and held for decapitation and fly emergence in the same manner as above.

Ant bodies that could be matched definitively with heads (i.e., head partially attached to body) were individually examined for *K. solenopsae* spores by microscopy to determine whether flies developed in infected ants. Heads that could not be definitively matched with bodies were held for emergence separately from heads from infected bodies. Samples of unmatched bodies (n = 59–66/colony) were individually examined for spores to estimate infection rates. *P. obtusus* that emerged from matched and unmatched heads were sexed and grouped into samples of 10–25 and 6–25 individuals, respectively and tested for the presence of *K. solenopsae* by PCR as described below. Hovering or ovipositing flies were collected and tested for *K. solenopsae* presence to test for potential vectoring capabilities or venereal transmission. Procedures followed those described for *V. invictae* exposures. The percentage of adult flies that successfully emerged from decapitated heads associated with infected bodies was determined.

An additional exposure of *K. solenopsae*-infected *S. invicta* to *P. obtusus* was made 2 months later using the methods described above. Infection rate of the isolated worker ants averaged 58%/colony (range 35–75%, n = 20 ants/colony), with two of three colonies used from the previous exposure. Because fly densities were high, ants were exposed to flies for only 24 h. Flies that emerged from ant heads that could be matched definitively with infected ant bodies were collected and either frozen or preserved in 95% ethanol for further examination. Emergence rate of adult flies also was determined for heads that were matched with infected bodies. Frozen adult *P. obtusus* (n = 30) were examined under phase-contrast microscopy at 400× for *K. solenopsae* spores in individual wet mounts. In addition, eight *P. curvatus* females were also examined for spores. The *P. curvatus* were apparently contaminants in the *P. obtusus* culture. Flies also were collected from unmatched heads, pooled into samples of 1, 3 or 5 flies by ant colony and sex, and examined for *K. solenopsae* by PCR (n = 20 samples).

Small-sized (1 g ≈ 1800 ants) workers from the same *K. solenopsae*-infected colonies described in the preceding paragraph were exposed for 1 day to another species of phorid fly, *Pseudacteon cultellatus* Borgmeier. For this exposure, heads were not matched with infected bodies. Adult flies that successfully emerged were pooled into three samples of five flies each for each ant colony and gender of fly. *K. solenopsae* presence was tested in a total of 18 samples by PCR. Emergence rate of adult flies from decapitated heads was determined.

Pseudacteon flies were collected in two locations in a pasture in Levy County, Florida where *K. solenopsae* was present in about 40% of the nests. Arbitrarily selected *S. invicta* nests were disturbed by inserting a shovel and creating a small cavity. Flies were aspirated as they hovered over disturbed workers. Flies were taken to the laboratory alive, anesthetized with CO_2, identified to species and sexed before being preserved in 95% ethanol. The majority of the flies caught were *P. curvatus* females (n = 164). Males of this species are rarely found at *S. invicta* nests (Wuellner et al., 2002) so they were not tested. DNA was extracted from 12 pooled groups of *P. curvatus*, with 10–12 flies/group, and examined by multiplex PCR. In addition, 10 female and 10 male *P. tricuspis* were also examined in three pooled groups of six or eight flies/group.

2.3. PCR detection of V. invictae and K. solenopsae

DNA was extracted from *Pseudacteon* flies by the method of Valles et al. (2002). Briefly, flies (2–10) were homogenized in 150 μl of

lysis buffer (50 mM Tris–HCl, pH 8, 4% sodium dodecyl sulfate and 5% β-mercaptoethanol) in a 1.5 ml microcentrifuge tube with a disposable plastic pestle for 15 s and the mixture was incubated at 100 °C for 15 min. The mixture was allowed to cool on ice for 1 min, followed by the addition of 200 µl of phenol:chloroform:isoamyl alcohol (Tris–HCl-saturated, pH 8). The mixture was mixed by inversion and centrifuged at room temperature for 5 min at 16,000*g*. The supernatant was removed and nucleic acids precipitated with ice-cold isopropanol and the pellets were washed twice with 70% ethanol. Pellets were dried at 37 °C, and resuspended in 30 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8).

Detection of *K. solenopsae* and *V. invictae* in *Pseudacteon* flies was accomplished by PCR. Examination of *P. obtusus* for *V. invictae* was conducted as described by Valles et al. (2004). Briefly, oligonucleotide primers p90 (5′-CACGAAGGAGGATAACCACGGT) and p93 (5′-CGCAATCAGTCTGTGAATCTCTTCA), specific for the *V. invictae* 16S small subunit ribosomal DNA gene were used to detect *V. invictae*. DNA integrity was verified by subsequent PCR analysis with oligonucleotide primers for conserved regions of phorid fly 18S ribosomal subunit (P800: 5′-GTAGTACACCTATACATTGGGTTCGTAC ATTACTCTA and P801: 5′-ATAAGTTTCAACGCTATAATCCTGAAAG CATC).

For *K. solenopsae* detection, multiplex PCR was conducted (Valles et al., 2002). Oligonucleotide primers specific to the small subunit ribosomal DNA gene (Moser et al., 1998, 2000; Valles et al., 2002; Accession No.: AF031538) of *K. solenopsae* (P1: 5′-CGAAGCATGAA AGCGGAGC and P2: 5′-CAGCATGTATATGCACTACTGGAGC) and the *Gp-9* gene (Krieger and Ross, 2002; Valles and Porter, 2003; Accession No.: AF459414) of *S. invicta* (P7: 5′-TAAAATTCCAAATCTAGG CTTTCGC and P8: 5′-CAAACATGAGAGTGCAGTGTGAACA) were included in the same reaction. PCR for the detection of the microsporidia was conducted by the hot start method in a PTC 100 thermal cycler (MJ Research, Waltham, MA). *K. solenopsae*-specific thermal cycling was as follows: 1 cycle at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 55 °C for 15 s and 68 °C for 30 s, followed by a final elongation step of 5 min at 68 °C. *V. invictae*-specific thermal cycling was conducted as follows: 1 cycle at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 55 °C for 15 s and 68 °C for 45 s, followed by a final elongation step of 5 min at 68 °C. The reactions were conducted in a 25 µl volume containing 2 mM $MgCl_2$, 200 µM dNTP mix, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 µM of each primer and 50 ng of the genomic DNA preparation. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. For all experiments, positive and negative controls were run alongside treatments. Presence of *K. solenopsae* DNA yielded a 318 nucleotide band while *V. invictae* DNA would yield a 791 nucleotide band.

Fly samples testing positive by PCR for *K. solenopsae* were re-amplified with oligonucleotide primers specific for three different *K. solenopsae* genes. Amplicons from these reactions were cloned and sequenced to verify their identity. The three *K. solenopsae* genes (and oligonucleotide primers used to amplify them) included the 16S rDNA gene (P1 and P2, Genbank Accession No.: AF031538), an uncharacterized integral membrane protein gene (P383: 5′-GCG GAAACCAGCCATAAAAGAGGCGGATGA and P386: 5′-CCTGCTTTTT ATCTGCAAGCATAGACATTGGTGTAAGTT, Genbank Accession No.: EH413084) and a 14–3–3 protein gene (P377: 5′-GAGCAGAAAGATA TGAAGAAATGGTGAAAGGAGTCAA and P380: 5′-ATATTCAACGCTTC TTCGCAAATATCACACAATTCC, Genbank Accession No.: EH413075; Valles et al., 2008). Amplification conditions with the integral membrane protein- and 14–3–3 protein-specific oligonucleotide primers were as follows: 1 cycle at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 67 °C for 15 s and 68 °C for 30 s, followed by a final elongation step of 5 min at 68 °C. Amplicons were ligated into the pCR4-TOPO vector, transformed into TOP10 competent cells (Invitrogen) and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida).

3. Results

3.1. Vairimorpha invictae exposure

Vairimorpha invictae was not detected in any of the *P. obtusus* adults that were reared ($n = 25$) or most likely reared ($n = 33$) from *V. invictae*-infected *S. invicta* workers. Similarly, there was no evidence of *V. invictae* infection in flies that emerged from head capsules that could not be matched with their bodies ($n = 334$, Table 1). The infection rate of the unmatched ant bodies, which was estimated from matched and unmatched headless ants was 87% (82/94). Thus, a large number of the 334 pupae from unmatched heads almost certainly came from infected workers. *V. invictae* also was not detected in hovering or ovipositing flies collected during fly access to infected *S. invicta* (Table 2). Hence, there was no evidence for *P. obtusus* mechanically vectoring *V. invictae* or for mechanical venereal transmission.

3.2. Kneallhazia solenopsae exposure

Pseudacteon obtusus flies that emerged from *K. solenopsae*-infected *S. invicta* worker ants tested positive for *K. solenopsae* by PCR (Fig. 1); an amplicon of anticipated size (318 nucleotides with *K. solenopsae* rDNA small subunit-specific oligonucleotide primers) was observed in 80% of pooled groups in the first trial and 35% in the second trial (Table 3). *K. solenopsae* was also detected in 44% of the pooled samples of *P. cultellatus* (Table 4). No corresponding amplification was observed with the ant-specific (*Gp-9* gene) oligonucleotide primers indicating that contamination of the flies with ant (or *K. solenopsae*) DNA did not occur. No amplification was observed in the negative control and *S. invicta*-specific and *K. solenopsae*-specific amplicons were generated in the positive control further indicating that the assay was free of contamination and working properly. *K. solenopsae* was not detected in *P. obtusus* collected in attack trays during exposure to infected *S. invicta* workers in the laboratory (Table 2). The percentage of *P. obtusus* adults that emerged from heads associated with *K. solenopsae*-infected *S. invicta* bodies was 94.8% (91/96) for the first trial and 92.8% (142/153) for the second trial. *K. solenopsae* was detected in 75% of the bodies matched with decapitated heads in trial 1 and 69% in trial 2. The emergence rate for *P. cultellatus* was 70.4% (145/206) from head capsules that were not specifically associated with infected ant bodies.

One of the *S. invicta* colonies (colony #26) used in the *V. invictae* exposures was also infected with *K. solenopsae*, having 46.5% (20/

Table 1
Number of *Pseudacteon obtusus* with *Vairimorpha invictae* that emerged from *Solenopsis invicta* heads that were associated with *V. invictae*-infected ant bodies.

Association of ant heads and bodies	Samples with *V. invictae*/no. samples tested (total no. of flies)		
	Female	Male	Total
Flies from heads matched with infected ant bodies	0/2 (17)	0/1 (8)	0/3 (25)
Flies from heads near infected ant bodies	0/2 (26)	0/1 (7)	0/3 (33)
Flies from unmatched heads with a 77% infection rate[a]	0/8 (162)	0/4 (81)	0/12 (243)
Flies from unmatched heads with a 96% infection rate[b]	0/3 (59)	0/2 (32)	0/5 (91)

7–21 flies per sample.
[a] Infection rate based on subsample of 44 headless ant bodies (colony #26).
[b] Infection rate based on subsample of 47 headless ant bodies (colony #49).

Table 2
Number of infected pooled samples of *Pseudacteon obtusus* collected while hovering near and/or within trays containing *Vairimorpha invictae* and/or *Kneallhazia solenopsae*-infected *Solenopsis invicta*.

Pathogen and % infection of *S. invicta* (avg ± SD) exposed	No. infected samples/total no. of samples[a] (total flies)	
	Female	Male
V. invictae (85 ± 0)	0/4 (59)	0/3 (29)
K. solenopsae (72 ± 8)	0/4 (46)	0/2 (79)

[a] 9–15 flies per sample.

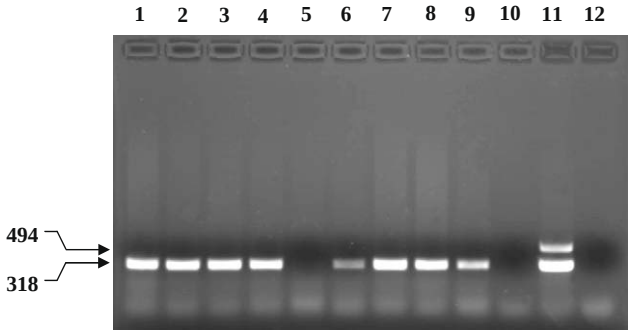

Fig. 1. Representative agarose gel separation of amplicons generated from multiplex PCR detection of the *Kneallhazia solenopsae* 16S gene (318 nucleotides) and the *Solenopsis invicta Gp-9* gene (494 nucleotides) using DNA isolated from *Pseudacteon obtusus* adult flies developing in *K. solenopsae*-infected *S. invicta* workers as template. DNA samples in lanes 1 through 10 were from pooled groups of flies ($n = 10$). Lanes 1, 5 and 10 were from female flies and lanes 2–4 and 6–9 were from male flies. Lane 11 is a positive control (*S. invicta* infected with *K. solenopsae*) and lane 12 is a negative template control. The 318 nucleotide amplicons represent positive detection of *K. solenopsae* (which was confirmed by sequence analysis) and the 494 nucleotide amplicon was positive detection of the *S. invicta Gp-9* gene.

Table 3
Number of pooled samples of *Pseudacteon obtusus* adults with *Kneallhazia solenopsae* that emerged from *K. solenopsae*-infected *Solenopsis invicta workers* from two trials.

Association of ant heads and bodies	Samples with *K. solenopsae*/no. samples tested (total flies)	
	Female	Male
First trial[a]		
Flies from heads matched with infected ant bodies	2/2 (22)	2/2 (50)
Flies from unmatched heads with a 65% infection rate[b]	1/2 (16)	2/4 (73)
Flies from unmatched heads with a 80% infection rate[b]	2/2 (26)	3/3 (61)
Flies from unmatched heads with an 70% infection rate[b]	0/1 (11)	4/4 (80)
Second trial[c]		
Flies from unmatched heads with a 35% infection rate[b]	1/3 (7)	0/4 (12)
Flies from unmatched heads with a 75% infection rate[b]	3/4 (12)	1/4 (12)
Flies from unmatched heads with an 65% infection rate[b]	0/1 (3)	2/4 (12)
Total (both trials)	9/15 (97)	14/25 (300)

[a] 6–25 flies per sample.
[b] Infection rate based on 20 individuals per colony obtained from ants selected for exposure to flies.
[c] 1, 3 or 5 flies per sample.

43) of the decapitated ants infected with *K. solenopsae*. DNA extracted from fly samples used in the *V. invictae* exposure was subsequently tested for *K. solenopsae* resulting in four of 12 samples positive for *K. solenopsae.* Among field-collected flies, *K. solenopsae* was detected in 17% of 12 pooled groups of *P. curvatus*, but not detected in three groups of *P. tricuspis*.

Blast (Altschul et al., 1997) analysis of the sequences from the *K. solenopsae*-positive flies indicated significant identity (99.1–100%

Table 4
Number of samples of *Pseudacteon cultellatus* adults with *Kneallhazia solenopsae* that emerged from *K. solenopsae*-infected *Solenopsis invicta*.

Association of ant heads and bodies	Samples with *K. solenopsae*/no. samples tested[a] (total flies)	
	Female	Male
Flies from unmatched heads with a 35% infection rate[b]	0/2 (10)	1/3 (15)
Flies from unmatched heads with a 75% infection rate[b]	3/4 (20)	3/3 (15)
Flies from unmatched heads with an 65% infection rate[b]	1/3 (3)	0/3 (12)
Total	4/9 (33)	4/9 (42)

[a] 1, 3 or 5 flies per sample.
[b] Infection rate based on 20 individuals per colony obtained from ants selected for exposure to flies.

for *P. curvatus*; 99.1–99.7% for *P. obtusus*) with the small subunit rDNA gene of *K. solenopsae* (Accession No.: AF031538). These data confirm that the amplicons generated from *P. curvatus* and *P. obtusus* were from *K. solenopsae*. Indeed, amplification was accomplished with oligonucleotide primers specific for two additional *K. solenopsae* genes (integral membrane protein and 14–3–3 protein) from *P. curvatus* and *P. obtusus*; these sequences were also identical to *K. solenopsae* (data not shown).

4. Discussion

This is the first report of a fire ant-infecting pathogen being detected in parasitoid flies of fire ants. Previous tests with a fire ant virus in *Pseudacteon* flies were negative (Valles and Porter, 2007). In the laboratory, *P. obtusus* that developed in *K. solenopsae*-infected *S. invicta* workers acquired the microsporidium in the majority of the pooled samples. *K. solenopsae* was not detected in any of the hovering or ovipositing flies, indicating that there was no mechanical acquisition of the microsporidium during oviposition and that flies were not infected prior to exposure to the infected fire ants. *K. solenopsae* was also found in two other species of fly, *P. cultellatus* in laboratory exposure and *P. curvatus*, which was collected in a field site where *K. solenopsae* was present.

To estimate the individual infection rate from the observed proportion of negative pooled samples (Table 3), we used the following equation: $X = 1 - P^{1/n}$, where X = the estimated individual infection rate; P = the proportion of negative pooled samples; n = mean number of individuals per pooled sample. This equation is based on the principle that the probability of a negative event occurring n consecutive times is the product of the probability of each individual event. The nth root of P is the probability of the individual event. The results of this equation are an approximation because n is a mean rather than a constant number. The proportion of positive individuals in *P. obtusus* in the first laboratory trial was estimated to be approximately 9% ($1-[0.20^{1/17}]$, based on a mean of 17 flies per pooled sample [range 6–25], with 20% of samples being negative and assuming no false negatives). In the second trial with *P. obtusus*, two of 10 individual flies were positive (20%) and five of 10 pooled samples of 3–5 flies were negative indicating about a 13% positive rate. ($1-[0.50^{1/4.8}]$), based on a mean of 4.8 flies per sample and 50% of pooled groups being negative). Since about 70% of the ants were infected (Table 3) and only 9–20% of the flies were positive, it appears that most of the flies developing in infected ants do not acquire the pathogen. An alternate explanation is that most of the fly larvae developing in infected workers do not survive. While this cannot be ruled out entirely, fly production from the infected fire ant workers was equivalent to that normally seen with uninfected workers, and the percentage of infected ant bodies associated with fly puparia

in heads (71%) was about the same as the percentage of exposed workers that were infected (65%).

Contrary to findings of *K. solenopsae* in *Pseudacteon* flies, *V. invictae* was not detected in any of the *P. obtusus* that were reared from *V. invictae*-infected ants nor from flies that were collected during the exposure to infected ants. Similarly, in Buenos Aires, Argentina, *V. invictae*-infected colonies of *Solenopsis richteri* Forel were collected and exposed to field populations of *Pseudacteon* flies. *V. invictae* was not detected by PCR in either flies collected flying above the *S. richteri* or from adult flies reared from the exposed colonies. However, *V. invictae* infection levels were reported to be very low in the exposed colonies (Calcaterra and Briano, 2005). Evidence of *K. solenopsae* but not *V. invictae* in flies reared from workers infected with both microsporidia suggests different life cycles and host interactions between the microsporidia and the flies. Dedeine et al. (2005) found *Wolbachia* variants in several species of *Pseudacteon* flies. However, all of the variants in the flies were different from the *Wolbachia* variants found in their fire ant hosts. Hence, there was no evidence of *Wolbachia* transmission between these fire ant parasitoids and host.

Whether fly cells are actually infected by *K. solenopsae* or if spores are simply being ingested by the flies during larval development needs further investigation. *K. solenopsae* has been detected by PCR in the heads of adult fire ants (SMV, unpublished data) and spores of both *K. solenopsae* (free spores, meiospores and megaspores) and *V. invictae* (free spores and meiospores) have been observed by microscopy in their heads (DHO, unpublished data). Thus, spores are available for ingestion by fly larvae and *K. solenopsae* megaspores have been observed in whole body wet mounts of *P. obtusus* (DHO, unpublished data). Accordingly, infection appears to be a likely event because *K. solenopsae* is detected and *V. invictae* is not. If ingestion of spores was causing positive detection, both microsporidia would be expected to be detected. Microsporidia have been reported to infect parasitoid cells (direct infection) after developing in infected hosts (Andreadis, 1980; Own and Brooks, 1986; Brooks, 1993; Schuld et al., 1999; Futerman et al., 2006). In addition, indirect infection has been documented, where non-digestible spores accumulated in the alimentary canal. This has been reported in immature, parasitic tachinid flies (Cossentine and Lewis, 1986), as well as in several parasitic hymenoptera (Brooks, 1993; Hoch et al., 2000). In both direct and indirect infections, parasitoids were negatively impacted, including one or several effects such as higher larval mortality, reduced adult emergence, shorter adult longevity and reduced fecundity (Brooks, 1993; Own and Brooks, 1986; Siegel et al., 1986; Futerman et al., 2006). *P. obtusus* emergence from infected *S. invicta* was greater than 92%. While this was higher than the usual emergence rate of 85%, it was within the range of *P. obtusus* emergence in laboratory reared flies, and development time was also normal (SDP, unpublished data). This suggested that parasitoid development during the pupal stage was not negatively impacted when the host was infected.

Parasitoid vectoring of microsporidia via oviposition, either by a contaminated ovipositor, or possibly by injection of spores has been reported in hymenopteran parasitoids (Brooks, 1993; Becnel and Andreadis, 1999). The possibility of *Pseudacteon* flies transmitting *K. solenopsae* is an important aspect that we plan to examine in the future. Field transmission of *K. solenopsae* to fire ant colonies by the transfer of infected fire ant brood has been demonstrated in non-territorial, polygyne fire ant colonies (Williams et al., 1999; Oi and Williams, 2003; Oi et al., 2008). However, Valles and Briano (2004) reported nearly half of surveyed Argentine fire ant colonies with *K. solenopsae* were monogyne ($n = 20$), whereas in the USA natural sustained infections in monogyne populations are infrequent (Oi et al., 2004; Milks et al., 2008; Fuxa et al., 2005; Oi and Valles, 2008). Field transmission by *Pseudacteon* flies could potentially explain, at least partially, this discrepancy between polygyne and monogyne infection rates between Argentina and the USA, because decapitating flies are still much more common in South America than they are in the USA. Another possible route of infection that needs to be investigated is the consumption of flies with *K. solenopsae* spores that were scavenged or captured by fire ants. In the laboratory, 60–90% of flies die or are captured after only a few hours of attacking fire ant workers. Discovery of a natural vector of *K. solenopsae* would advance the understanding of the life cycle and epidemiology of *K. solenopsae* and possibly improve its utilization as a biological control agent for fire ants.

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