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Research Article

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Oviposition Behavior and Survival of *Tamarixia radiata* (Hymenoptera: Eulophidae), an Ectoparasitoid of the Asian Citrus Psyllid, *Diaphorina citri* (Hemiptera: Liviidae), on Hosts Exposed to an Entomopathogenic Fungus, *Isaria fumosorosea* (Hypocreales: Cordycipitaceae), Under Laboratory Conditions

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Abstract

Antagonistic interactions between the nymphal parasitoid, *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), and the ARSEF 3581 strain of the entomopathogenic fungus, *Isaria fumosorosea* Wize (Hypocreales: Cordycipitaceae), could disrupt biological control of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Three interactions were evaluated under laboratory conditions at 25 °C: 1) parasitoid survival if parasitized hosts were exposed to ARSEF 3581 blastospores before or after host mummification; 2) parasitoid survival if mummies containing larva or pupa were exposed to ARSEF 3581 hyphae; 3) parasitoid oviposition on infected hosts with visible or without visible hyphae. Topical application of blastospore formulation onto the dorsal surfaces of live nymphs parasitized with second-instar wasp larva (3 d after parasitism) reduced host mummification by 50% and parasitoid emergence by 85%. However, parasitoid emergence was not affected by topical application of blastospore formulation onto mummies that contained fourth-instar wasp larva (6 d after parasitism). Parasitoid emergence was reduced by 80% if mummies containing fourth-instar wasp larva were covered with blastospore formulation colonized by fungal hyphae. In comparison, parasitoid emergence was not affected if mummies containing wasp pupa (9 d after parasitism) were covered with formulation colonized by fungal hyphae. Female parasitoids oviposited on infected hosts without visible hyphae but not on infected hosts with visible hyphae. Our findings suggest that *I. fumosorosea* could detrimentally affect *T. radiata*, if both natural enemies are simultaneously deployed for biological control of *D. citri*. However, temporal separation of the fungus and parasitoid could reduce antagonism and enhance control of *D. citri*.

Key words: biological control, *Diaphorina citri*, entomopathogen, *Isaria fumosorosea*, Tamarixia radiata

Antagonistic interactions among natural enemies can disrupt biological control strategies for phytophagous pest insects (Rosenheim et al. 1995, Snyder and Ives 2008, Straub et al. 2008, Letourneau et al. 2009). If parasitoids and entomopathogenic fungi use the same insect species for hosts, then spatial and temporal overlap can lead to competition and intraguild predation (Polis and Holts 1992, Roy and Pell 2000, Furlong and Pell 2005). Understanding the causes behind antagonistic interactions among insect parasitoids and entomopathogenic fungi is crucial to successful integration of these natural enemies in area-wide pest management programs for the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), a key pest of citrus worldwide (Hall et al. 2012a, Grafton-Cardwell et al. 2013).

Adult and nymphal stages of *D. citri* acquire and vector two species of phloem-limited bacterium, *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus, that are putatively the causative agents of huanglongbing (HLB) or citrus greening disease (Halbert and Manjunath 2004, Gottwald 2010, Pelz-Stelinski et al.

2010). Symptoms of citrus trees infected with HLB include inferior fruit quality, fruit drop, yield reduction, and eventually tree death (Bové 2006). This insect vector and plant pathogen complex is the most serious threat that the US citrus industry currently faces (Grafton-Cardwell et al. 2013).

In California, Florida, and Texas, a broad range of citrus and rutaceous host plants for *D. citri* and HLB are used for ornamental or landscape plantings (Damsteegt et al. 2010, Sétamou et al. 2016). Most of Florida's citrus-producing regions are not located near urban areas, but California and Texas share a pressing problem with *D. citri* and HLB spreading in residential landscapes near citrus groves (Sétamou et al. 2012, Richards et al. 2014). Insecticidal treatment of residential psyllid-infested trees is costly and problematic. In fact, urban spray programs for *D. citri* in southern California were largely abandoned because of high treatment costs, ~US$100 per residence, and problems in accessing residential properties (Hoddle and Pandey 2014). Therefore, it is generally accepted that control of *D. citri* in urban areas will need to rely heavily on biological control by native or introduced natural enemies such as predators, parasitoids, or entomopathogens (Hall et al. 2012a, Grafton-Cardwell et al. 2013).

Natural enemies currently available for biological control of *D. citri* in the United States include a native entomopathogenic fungus, *Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae), two exotic parasitoids, *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), and several species of coccinellid beetles, syrphid flies, lacewings, and spiders (see Michaud 2004, Qureshi and Stansly 2009 for reviews of key predators of *D. citri*). Various isolates of *I. fumosorosea* (*Ifr*) have been used to control a wide range of pest insects (see Zimmermann 2008 for a review of *Ifr* biology, ecology, and use in biological control), and *Ifr* was isolated from mycosed *D. citri* found on orange trees in Florida (Meyer et al. 2008, Hoy et al. 2010). Since 2014, *D. aligarhensis* has been released in California, but not Texas and its establishment is still being evaluated (Bistline-East et al. 2015). In comparison, *T. radiata* is currently considered an important agent for classical biological control of *D. citri* in both California and Texas (Flores et al. 2014, Kistner et al. 2016).

Adult females of *T. radiata* are solitary and synovigenic ectoparasitoids that parasitize and host feed on third- to fifth-instar nymphs of *D. citri* (see Chen and Stansly 2014 for a review of *T. radiata* biology and ecology). Developing *T. radiata* larvae feed externally on parasitized nymphs, kill and mummify their hosts, and emerge as wasps. Since 2011, *T. radiata* from Pakistan have been mass-reared and released in mainly urban areas throughout southern California and the Lower Rio Grande Valley (LRGV) of Texas (Flores et al. 2014, Kistner et al. 2016). In urban areas of California and Texas where *T. radiata* has become established, parasitoid-attributed mortality of *D. citri* can be high during the fall when populations of psyllid nymphs and parasitoids peak, but it can also be low during other seasons when nymphs or parasitoids are scarce. Coordinated use of both *T. radiata* and *Ifr* could enhance suppression of *D. citri*, especially during periods when psyllid populations consist primarily of adults or when *T. radiata* populations are low.

Several native entomopathogenic fungi naturally infect *D. citri* in the United States (Meyer et al. 2007, 2008; Hall et al. 2012b), but interest has centered on two *Ifr* strains, the United States Department of Agriculture (USDA)–Agricultural Research Service Entomopathogenic Fungi (ARSEF) 3581 and the Apopka 97 (American Type Culture Collection 20874) used for the PFR 97 20% WDG microbial insecticide (Certis Inc., Columbia, MD). Both strains are highly pathogenic toward adult and nymphal stages of *D. citri* and easily mass-produced as blastospore formulations (Jackson et al. 1997; Avery et al. 2009, 2011; Stauderman et al. 2012) for use in foliar applications or autodissemination management strategies. "Autodissemination" is the dispersal of an entomopathogen by an insect to conspecifics (Vega et al. 2007), and *Ifr* blastospores are ideal for autodissemination by *D. citri* because they are desiccation tolerant and germinate quickly on suitable hosts (Jackson and Payne 2007, Jackson et al. 2010, Moran et al. 2011).

Patt et al. (2015) showed the potential for using autodisseminators (dispensers) to attract and then inoculate *D. citri* adults with ARSEF 3581 blastospores for the purpose of inducing epizootics in residential citrus trees. They found that in greenhouse trials, exposure of free-flying adult psyllids to autodisseminators resulted in an average of 56% of the insects becoming contaminated or autoinoculated with blastospores. In addition, the contaminated adults in turn transferred blastospores to exposed psyllid colonies and infected ~35% of the nymphs. Since ARSEF 3581 is a south Texas strain well adapted to the LRGV (Humber 2011), autodissemination of ARSEF 3581 blastospores could be combined with inoculative releases of *T. radiata* to enhance control of *D. citri* on residential citrus in this region. Published studies on interactions between *T. radiata* and *Ifr* that could impact control of *D. citri* are currently lacking in the literature. Parasitoids and entomopathogenic fungi can directly compete on or within shared hosts, such as when the host is both parasitized and infected by a fungus (Fransen and van Lenteren 1994, Roy and Pell 2000, Mesquita and Lacey 2001, Furlong and Pell 2005, Avery et al. 2008). However, parasitoids may evade direct competition with entomopathogenic fungi by not ovipositing in or on infected hosts (Fransen and van Lenteren 1993, Mesquita and Lacey 2001, Rännbäck et al. 2015). Understanding behavioral and competitive interactions between *T. radiata* and *Ifr* will benefit programs seeking to integrate these two natural enemies for biological control of *D. citri*. Presently, it is unknown as to whether *T. radiata* will parasitize fungal infected hosts and how exposure of parasitized hosts to *Ifr* will affect parasitoid survival. The aims of this study were 1) to evaluate adult emergence of *T. radiata* from parasitized hosts exposed to ARSEF 3581 and 2) to determine whether *T. radiata* will oviposit on hosts infected by ARSEF 3581.

Materials and Methods

Plants

Orange jasmine, *Murraya exotica* (L.) Millsp cv. 'Lakeview', was used for rearing *D. citri*. Two- to three-year-old plants were grown in plastic pots (4 or 11 liter) with a sand-peat-perlite potting mixture and time-release fertilizer (Osmocote 18-7-10, The Scotts Company, Marysville, OH). Mature foliage of orange jasmine is unsuitable for oviposition by psyllid adults or development of psyllid nymphs; therefore, plants were pruned to promote production of young flush shoots. Insecticidal soap was used as needed to control pest insects or mites on the plants. A nonsystemic fungicide (1 ml concentrate per 1 liter water, Bravo Weather Stik, Syngenta Crop Protection LLC, Greensboro, NC) was applied onto plants as a foliar spray to prevent the development of saprophytic fungi within the psyllid colonies. However, we avoided possible detrimental effects of the fungicide on our study insects and *Ifr* by: 1) applying fungicide to plants at least 3 mo prior to their infestation with *D. citri*, 2) pruning the plants at least 2 wk prior to infestation with *D. citri* to remove most of the mature fungicide-treated foliage and produce new flush shoots that were never exposed to fungicide.

Insect Colonies

Colonies of *D. citri* and *T. radiata* were maintained by the USDA–Animal and Plant Health Inspection Service–Plant Protection and Quarantine–Center for Plant Heath and Technology (APHIS-PPQ-CPHST) laboratory in Mission, TX. The *D. citri* colony was started in 2007 with adults collected on citrus trees at Mission and has been maintained on orange jasmine. The *T. radiata* colony was started in 2009 with adults from a laboratory strain (DPI-PK) established by the Florida Department of Agriculture and Consumer Services—Department of Plant Industry from a collection on citrus in Punjab, Pakistan (Barr et al. 2009). The APHIS-PPQ-CPHST parasitoids were reared in screen cages (75 cm in width by 75 cm in depth by 115 cm in height, BugDorm-2400 Insect Rearing Tent, MegaView Science Co., Taichung, Taiwan) on *D. citri* nymphs infesting orange jasmine. Insect colonies were maintained at 28–30 °C, 40–60% RH, and a photoperiod of 14:10 (L:D) h inside greenhouses in Mission and Weslaco, TX.

Entomopathogenic Fungi

The ARSEF 3581 isolate of *Ifr* was cultured and the blastospore formulation was produced at the USDA–Agricultural Research Service National Center for Agricultural Utilization Research in Peoria, IL, by liquid culture fermentation as described in Jackson et al. (1997, 2003). Blastospores were harvested by adding pulverized cotton burrs (<60 mesh) to the blastospore culture to form a filter cake. The filter cake was air dried following methods given in Jackson and Payne (2007) to produce a formulation yielding 7.0–8.5 $\times$ 10^9 blastospores/g. Spore viability of the blastospore formulation ranged from 75–85% and was assessed by a 6-h germination assay used by Jackson et al. (1997). For the assay, suspensions of dried blastospore formulation in potato dextrose broth were incubated at 28 °C. After 6 h of incubation, 100 blastospores were examined for germ tube formation. A blastospore with a germ tube equal to or greater than half the length of the spore was counted as germinated and viable. Formulations were stored in vacuum-sealed polyethylene bags at 4 °C, which stabilized spore viability, for 1–3 wk prior to use.

Experimental Conditions

Vidal et al. (1997) found that *Ifr* isolates from the southern United States exhibited optimal growth at 25–28 °C. Chien et al. (1993) and Gómez-Torres et al. (2012) reported that 25 °C is the optimal temperature for development and survival of *T. radiata* on *D. citri*. Therefore, to optimize trial conditions for both the parasitoid and fungus, all experiments and preparation of insect or fungi stages were conducted in temperature-regulated rooms or growth chambers set at 25 °C with a photoperiod of 14:10 (L:D) h.

Leaf Disk Vials

For short-term rearing of parasitized nymphs before and after exposure to *Ifr*, we used leaf disk vials. Leaves of similar age and size were excised from the fully expanded flush of Rio Red grapefruit (*Citrus* $\times$ *paradisi* Macfadyen) trees maintained by the Texas A&M University-Kingsville Citrus Center, Weslaco, TX. The leaves were washed with a solution of distilled water and liquid soap (0.1 ml soap/2.0 liter water), rinsed with only distilled water to remove any soap residue, and wiped with paper towels to remove any remaining dirt or saprophytic fungi on their surface. Leaf disks (3.0 cm in diameter) were punched from the cleaned leaves. Each leaf disk was placed, with its abaxial surface facing up, inside a plastic snap-cap (2.5 cm in diameter by 0.5 cm in height) lined with a paper towel disk (2.5 cm in diameter) saturated with distilled water. A clear-plastic, 9-dram vial (2.5 cm in diameter by 6.7 cm in length, Thornton Plastics Co, Salt Lake City, UT) was inserted into the cap to make a sealed enclosure (Fig. 1) that could maintain high relative humidity (80–100%) to preserve the leaf disk and promote germination of *Ifr* blastospores. Leaf disk vials could be used immediately for rearing psyllid nymphs and vials with nymphs were held in a growth chamber inverted to expose the leaf disks to light.

Collection of *T. radiata* Larvae and Pupae

To obtain different larval and pupal stages of *T. radiata*, fifth-instar *D. citri* were exposed to female wasps of various ages in cylinder cages (Fig. 2). Fifth-instars were used because survival and emergence of *T. radiata* is highest on this host stage (Chien et al. 1991, Gómez-Torres et al. 2012). Shoots of orange jasmine flush infested with ~20–40 fifth-instars were put in florist water tubes (1.5 cm in diameter by 7.6 cm in length, Aquatube #54-97-00, Syndicate Sales Inc., Kokomo, IN) containing hydroponic solution (0.125 g of fertilizer per 1 liter of distilled water, MaxiGro 10-5-14 NPK Ratio, General Hydroponics, Sebastopol, CA). A single shoot in its water tube was inserted into a hole in the center of a wooden disk, which held the shoot upright. The open end of a clear-polypropylene cylinder (4 cm in diameter by 30 cm in length) was fitted around the wooden disk to enclose the shoot. The top of each cylinder was sealed with nylon mesh after three female wasps (3–5 d old) were released near the psyllid-infested shoot. The female wasps were removed from the cylinder cage after 24 h of confinement with the psyllid-infested shoot.

Cylinder cages described above were held in a temperature regulated room and the *D. citri* nymphs in each cage were examined for parasitism within 24 h of exposure to female wasps. Parasitism was determined by lifting nymphs off the shoots and using a stereomicroscope to examine their ventral sides for a *T. radiata* egg. Female *T. radiata* rarely superparasitize and usually oviposit only one egg next to one of the hind coxa of a nymph (Fig. 3). All parasitized nymphs were transferred to leaf disk vials and checked every 24 h to assess whether the immature *T. radiata* had developed into the larval or pupal stages required for experiments.

Fig. 1. Leaf disk vial used for short-term rearing of psyllid nymphs before and after exposure to *Ifr*.

Fig. 2. Cylinder cage used for confining adult females of *T. radiata* with psyllid-infested shoots of orange jasmine.

Fig. 3. A single *T. radiata* egg inserted behind the left hind coxa of a psyllid nymph.

Preparation of *I. fumosorosea* Hyphae

To obtain fungal hyphae for our trials, we used the same vial setup described above for leaf disks except 0.01–0.02 g of granulated ARSEF 3581 formulation was weighed out on a microbalance and then placed directly on the paper towel disk. Vials with formulated granules were incubated in a growth chamber. The nutrient-rich granules enabled extensive hyphal proliferation after blastospore germination. Within 4–8 d of incubation, the granules were covered with *Ifr* hyphae (mycelium) and used for experiments. Under our experimental conditions, we did not observe conidia or conidiaphores on the granules within 4–8 d of incubation.

Preparation of *I. fumosorosea*-Infected *D. citri* Nymphs for Exposure to *T. radiata*

To obtain groups of *Ifr*-infected hosts for exposure to *T. radiata*, we dipped clusters of *D. citri* nymphs in a blastospore suspension (~7.0×10^6 blastospores per ml). Our dipping technique ensured that all the nymphs in each group were simultaneously inoculated. Stem bottoms of orange jasmine flush (8.0–10.0 cm in length) were inserted into 1.5-ml microcentrifuge tubes filled with distilled water. Each flush shoot was infested with 30 fifth-instar *D. citri*. Parafilm M film was wrapped around the top of each tube and the stem to prevent water and insect loss. Blastospore suspensions were prepared by agitating ARSEF 3581 formulation (1 g per 1,000 ml) in distilled water as described in Moran et al. (2011). A flush shoot and its entire nymph cluster was dipped and swirled for 60 s in a 1,000-ml beaker filled with blastospore suspension. Each dipped shoot was inserted into a clear-polypropylene, 50-ml conical tube (3.0 cm in diameter by 11.5 cm in length, Falcon #352098, Corning Science, Mexico). The tube opening was covered with Parafilm M film that was perforated for ventilation. Relative humidity inside conical tubes containing a single psyllid-infested shoot ranged from 72–87% (mean SEM = 78.5 ± 0.5%). Inoculated nymphs were incubated in a growth chamber for 0, 24, or 48 h before they were exposed to *T. radiata*.

Emergence of *T. radiata* From Live Hosts and Mummified Hosts Exposed to *I. fumosorosea*

Experiments were conducted to evaluate the level of adult wasp emergence when parasitized hosts were treated with *Ifr* at different times after parasitism. *Isaria fumosorosea* and *T. radiata* will directly compete if parasitized nymphs become infected by the fungus. For the first set of experiments, we evaluated parasitoid emergence when blastospores were applied either before or after host mummification. Two treatments were tested: 1) blastospores applied at 3 d after parasitism (live nymphs with second-instar wasp larvae; $n = 30$); 2) blastospores applied at 6 d after parasitism (mummified nymphs with fourth-instar wasp larvae; $n = 28$). For the second set of experiments, parasitoid emergence was evaluated following hyphal placement on mummies with either larvae or pupae of *T. radiata*. The following treatments were tested: 3) hyphae applied at 6 d after parasitism ($n = 24$), and 4) hyphae applied at 9 d after parasitism when mummies contained pupae of *T. radiata* ($n = 24$). For each trial, a parasitized nymph or a mummy was held in a leaf disk vial as described above and then treated with blastospores or hyphae.

For blastospore treatments, a synthetic-fiber paint brush was used to apply ARSEF 3581 formulation to the dorsal regions of the nymph or mummy. This inoculation technique simulated physical transfer of blastospores to psyllid nymphs or mummies from adult psyllids inoculated by autodisseminators (Patt et al. 2015). For hyphal treatments, a brush was used to surround a mummy with 0.01–0.02 g of *Ifr* formulation covered with mycelium. This second inoculation technique simulated exposure of mummies to hyphal growth from adjacent cadavers of mycosed psyllids. As controls for treatments 1–4, trials were conducted using untreated parasitized nymphs or untreated mummies during the same time as the treatment sets ($n = 30, 28, 24, 24$, for controls respectively).

Leaf disk vials with treated or control insects were held in a growth chamber (conditions as described above) for 16 d or until the emergence of adult wasps. Every 24 h, the leaf disk vials were opened for aeration and each nymph or mummy was checked for mycosis, mummification (only nymphs), and wasp emergence. The

total numbers and sex of adult wasps that emerged from each set of trials were recorded.

Parasitism of Hosts Infected by *I. fumosorosea*

The effects of *Ifr* infection on parasitism of *D. citri* nymphs by *T. radiata* were evaluated. For these experiments, the stage of fungal infection prior to exposing fifth instars to female wasps was manipulated. The experiments consisted of two parts. The first part evaluated parasitism after fungal hyphae was visibly extending from the bodies of hosts (48–72 h after inoculation). The second part evaluated parasitism before fungal hyphae became visible on hosts (0–48 h after inoculation). A stereo-microscope was used to confirm that fungal hyphae were not visibly extending from the bodies of inoculated nymphs if the insects were incubated for <48 h after being dipped in blastospore suspension.

To standardize female wasps for experiments, females of similar size and age (24 h old) were selected. *Tamarixia radiata* is a synovigenic parasitoid, which means that female wasps have very few eggs upon emergence and require host-feeding before they can mature eggs or produce additional eggs (Chen and Stansly 2014). To ensure that female wasps had mature eggs, they were allowed to feed on psyllid nymphs prior to testing. Individual female wasps were confined for 96 h in clear conical tubes with orange jasmine shoots infested with clusters of 30 to 50 second- to fourth-instar psyllids.

For the first experiment, each female wasp was confined in a conical tube with a psyllid-infested shoot and given one of two treatments over 3 d: control or fungus-treated hosts or only control hosts. "Fungus-treated hosts" consisted of *D. citri* nymphs dipped in a blastospore suspension and incubated for 48 h before testing. "Control hosts" consisted of nymphs dipped in only distilled water and incubated for 48 h. Females given "control or fungus-treated hosts" were offered 30 control hosts on day 1, then 30 fungus-treated hosts on day 2, and a different set of 30 control hosts on day 3. In comparison, females given "only control hosts" were offered different sets of 30 control hosts on days 1, 2, and 3.

In the second experiment, the same shoot bioassay was used but each wasp was presented on days 1 and 3 with 30 control nymphs and on days 2 and 4 with 30 fungus-treated nymphs. The first and second sets of control nymphs were dipped in distilled water and incubated for 0 and 24 h, respectively, while the first and second sets of fungus-treated nymphs were dipped in blastospore suspensions and incubated for 0 and 24 h, respectively.

For both experiments, parasitism in each tube was assessed within 24 h after wasp removal by using a stereo-microscope to inspect the ventral side of each nymph for parasitoid eggs. In the second experiment, all parasitized nymphs were transferred to leaf disk vials and held for 16 d or until adult wasps emerged. Each host was checked daily for mycosis, mummification, and wasp emergence. The total number and sex of wasps that emerged in each tube were recorded.

Female wasps were screened to minimize oviposition variation due to egg load. Only females that parasitized control hosts on day 3 were counted as treatment replicates. Twelve replicates for each of the three treatments (total = 36 wasps) were completed. To assess whether *T. radiata* females were infected after handling *Ifr*-treated hosts, we recovered the wasps and confined each one in a 9-dram plastic vial. Wasps died by starvation in the vials. For 12 consecutive days, each wasp was examined daily for mycosis. All wasps and parasitized nymphs were held in growth chambers throughout the experiments.

Statistical Analysis

All data analyses, except where noted, were performed using SigmaPlot 12.5 Exact Graphs and Data Analysis software package (Systat Software 2013). Fisher's exact test was used to compare host mummification (proportion of parasitized psyllid nymphs that mummified), wasp emergence (proportion of mummies from which adults of *T. radiata* emerged), and secondary sex ratio (proportion of males among adult wasps) among *Ifr*-treated hosts and controls. The VassarStats: Statistical Computation Website (http://vassarstats.net/) (accessed 19 October 2015) was used to perform the Freeman–Malton extension of the Fisher exact test for a "2 × 4" contingency table (Freeman and Halton 1951) to compare wasp emergence and secondary sex ratio across controls for different experiments. Parasitism (proportion of psyllid nymphs that *T. radiata* oviposited on) by female wasps given series of only control hosts or series of control hosts and fungus-treated hosts was analyzed by Friedman's test and if significant treatment effects were found, the Student–Newman–Keuls test was used for pair-wise comparisons of treatments (host types) within each series. The Mann–Whitney *U*-test was used to compare parasitism by female wasps given different series of hosts.

Results

Emergence of *T. radiata* From Live Hosts and Mummified Hosts Treated With Blastospores

Host mummification, wasp emergence, and secondary sex ratio were affected by the timing of blastospore treatments. Parasitoid survival was greatly reduced if *D. citri* nymphs, parasitized with second-instar *T. radiata*, were treated with blastospores. The percentage of mummies that developed in the untreated control group was more than two times higher than in the blastospore-treated group (Fig. 4). Within 5–6 d after treatment with blastospores, hyphae covered the nymph cadavers that failed to mummify. Within 16 d after treatment, cadavers of nymphs that failed to mummify and parasitoids that failed to emerge were completely colonized by *Ifr*. Emergence of *T. radiata* adults from the control group was seven times greater than emergence from the blastospore-treated group (Fig. 4). Of the 30 parasitized nymphs treated with blastospores, nine developed into mummies not visibly colonized by *Ifr*. From these nine mummies, only three adult male wasps emerged (Figs. 5a and 6a). In contrast, blastospore-treated

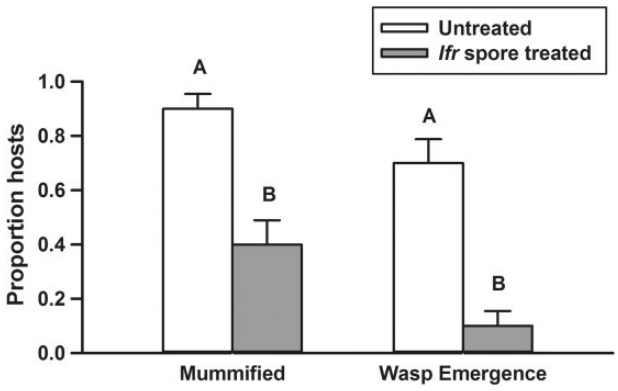

Fig. 4. Host mummification and wasp emergence after fifth-instar *D. citri* nymphs, carrying 3-d-old *T. radiata* (second-instar), were either treated with *Ifr* blastospores ($n = 30$) or untreated ($n = 30$). Pairs of columns with different letters are significantly different at $P < 0.01$ (Fisher's exact test; two-tailed). The standard error of proportion is provided for mummification and wasp emergence.

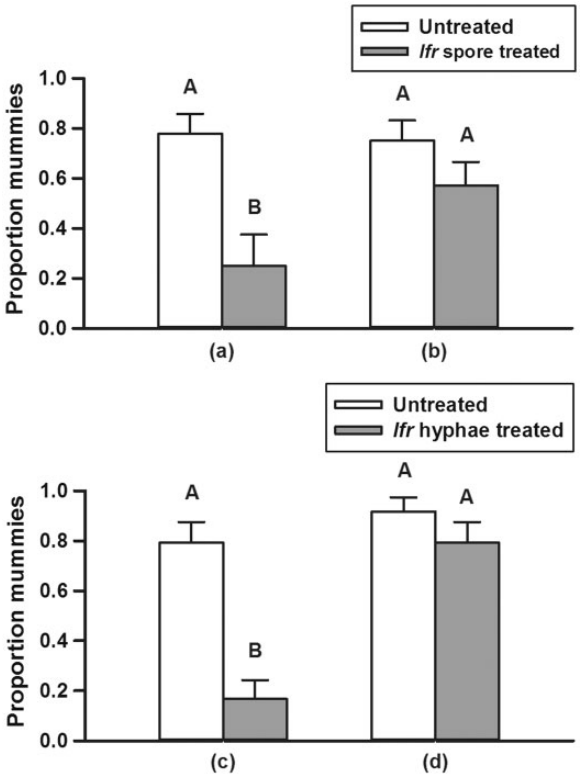

Fig. 5. Wasp emergence after live or mummified fifth-instar *D. citri* nymphs, carrying or containing *T. radiata* at different stages of development, were treated with either *Ifr* blastospores or hyphae: (**a**) 3-d-old *T. radiata* (second-instar) and treated with blastospores ($n = 12$ mummies from 30 live, parasitized nymphs) or untreated ($n = 27$ mummies from 30 live, parasitized nymphs), (**b**) 6-d-old *T. radiata* (fourth-instar) and treated with blastospores ($n = 28$ mummies) or untreated ($n = 28$ mummies), (**c**) 6-d-old *T. radiata* (fourth-instar) and treated with hyphae ($n = 24$ mummies) or untreated ($n = 24$ mummies), (**d**) 9-d-old *T. radiata* (pupa) and treated with hyphae ($n = 24$ nymphs) or untreated ($n = 24$ nymphs). Pairs of columns with different letters are significantly different at $P < 0.001$ (Fisher's exact test; two-tailed). The standard error of proportion is provided for wasp emergence.

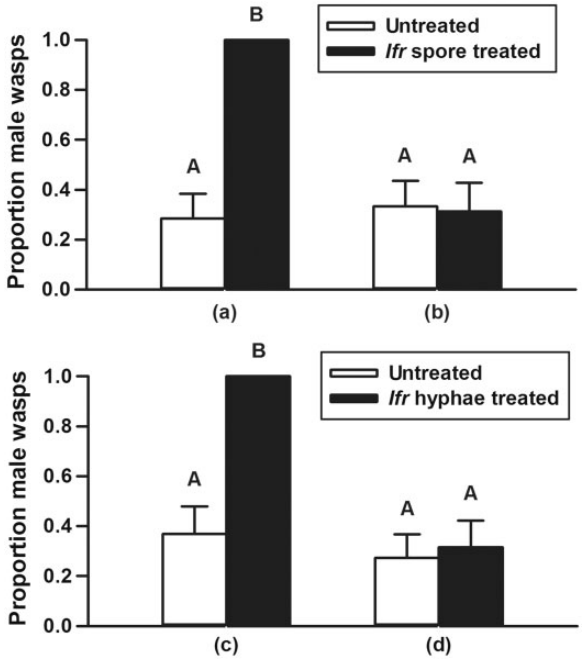

Fig. 6. Proportion of males among wasps that emerged after live or mummified fifth-instar *D. citri* nymphs, carrying or containing *T. radiata* at different stages of development, were treated with either *Ifr* blastospores or hyphae: (**a**) 3-d-old *T. radiata* (second-instar) and treated with blastospores ($n = 3$ wasps from 30 live, parasitized nymphs) or untreated ($n = 21$ wasps from 30 live, parasitized nymphs), (**b**) 6-d-old *T. radiata* (fourth-instar) and treated with blastospores ($n = 16$ wasps from 28 mummies) or untreated ($n = 21$ wasps from 28 mummies), (**c**) 6-d-old *T. radiata* (fourth-instar) and treated with hyphae ($n = 4$ wasps from 24 mummies) or untreated ($n = 19$ wasps from 24 mummies), (**d**) 9-d-old *T. radiata* (pupa) and treated with hyphae ($n = 19$ wasps from 24 mummies) or untreated ($n = 22$ wasps from 24 mummies). Pairs of columns with different letters are significantly different at $P < 0.05$ (Fisher's exact test; two-tailed). The standard error of proportion is provided for wasp emergence.

mummies were not colonized by *Ifr* and parasitoid survival was not reduced in this group. Emergence of *T. radiata* adults was similar among control mummies and blastospore-treated mummies (Fig. 5b). Sex ratios of adult wasps that emerged from control mummies or blastospore-treated mummies were female-biased and not statistically different (Fig. 6b).

Emergence of *T. radiata* From Mummified Hosts Treated With Hyphae

Adult wasp emergence was negatively affected among mummies containing parasitoid larvae treated with *Ifr* hyphae; however, this effect was not observed among mummies containing older parasitoid pupae. The percentage of adult wasps that emerged from control mummies was nearly five times higher than that recorded from hyphae-treated mummies containing fourth-instar *T. radiata* (Fig. 5c). However, wasp emergence from control mummies and hyphae-treated mummies with pupal *T. radiata* was similar (Fig. 5d). Only male wasps emerged from hyphae-treated mummies with larval *T. radiata* (Fig. 6c). Sex ratios of adult wasps that emerged from control mummies and hyphae-treated mummies with pupal *T. radiata* were female-biased and not statistically different (Fig. 6d). Survival and secondary sex ratio of *T. radiata* on control hosts were consistent throughout our experiments. Wasp emergence from control hosts among the four treatment groups was similarly high ($P = 0.427$; Freeman-Malton extension of the Fisher exact test; two-tailed) with a pooled value of 0.81 (83 wasps from 103 mummies because two of the 71 mummies that formed were damaged by handling). Sex ratios of adult wasps from control hosts were also not significantly different ($P = 0.923$; Freeman–Malton extension of the Fisher exact test; two-tailed) and the pooled sex ratio was female biased at 0.31 (26 males among 83 wasps).

Parasitism of Hosts Infected by *I. fumosorosea*

Mycosed nymphs were rejected by female wasps (Friedman's test: $\chi^2 = 20.667$; df = 2; $P < 0.001$; Fig. 7). On day 2, female wasps did not parasitize fungus-treated nymphs that were incubated for 48 h and exhibited mycosis. Instead, they apparently saved their eggs and parasitized, on average, three times as many control hosts on day 3 (mean ± SEM = 10.58 ± 1.49, $n = 12$ female wasps) than on day 1 (mean ± SEM = 3.17 ± 0.66, $n = 12$ female wasps. Student–Newman–Keuls test: $q = 3.266$; $P < 0.05$). Parasitism by female wasps given only control hosts increased by a smaller margin over the 3 d of host presentation (Friedman's test: $\chi^2 = 7.256$; df = 2; $P = 0.027$; Fig. 7). On day 1, they parasitized, on average, 50% fewer control hosts (mean ± SEM = 2.50 ± 0.52, $n = 12$ female wasps) than on day 2 (mean ± SEM = 5.08 ± 0.67, $n = 12$ female wasps. Student–Newman–Keuls test: $q = 3.464$; $P < 0.05$) or day 3

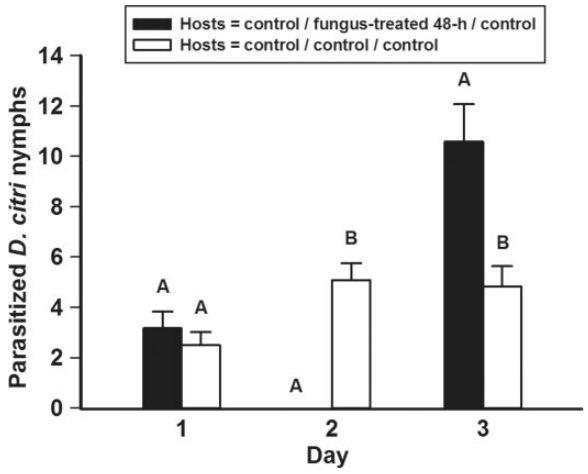

Fig. 7. Mean (+ SE) parasitism by individual females of *T. radiata* given only control hosts or control hosts and *Ifr*-treated hosts over three consecutive days. Control hosts were fifth-instar *D. citri* dipped in distilled water and incubated for 48 h. *Ifr*-treated hosts were fifth-instars dipped in an *Ifr* blastospore suspension and incubated for 48 h. In the control group, female wasps were exposed to different sets of 30 control nymphs on days 1, 2, and 3 ($n = 12$ wasps). In the test group, female wasps were exposed to 30 control nymphs on day 1, 30 infected nymphs on day 2, and a different set of 30 control nymphs on day 3 ($n = 12$ wasps). No *Ifr*-treated hosts were parasitized on day 2. Pairs of columns with different letters are significantly different at $P < 0.01$ (Mann–Whitney *U*-test).

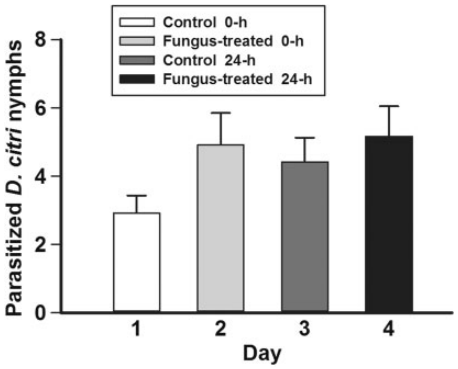

Fig. 8. Mean (+ SE) parasitism by individual females of *T. radiata* given different types of control nymphs and *Ifr*-treated hosts over four consecutive days. The control group consisted of fifth-instar *D. citri* dipped in distilled water and incubated for 0 or 24 h. The treatment group consisted of fifth-instars dipped in an *Ifr* blastospore suspension and incubated for 0 or 24 h. Parasitism of the four host types were not significantly different ($P = 0.138$; Friedman's test, $n = 12$ wasps).

(mean ± SEM = 4.83 ± 0.80, $n = 12$ female wasps. Student–Newman–Keuls test: $q = 3.674$; $P < 0.05$) but parasitism on days 2 and 3 were similar (Student–Newman–Keuls test: $q = 1.225$; $P > 0.05$). Fecundity of female wasps was similar in both treatment groups because the total numbers of nymphs parasitized over 3 d was similar between wasps given either only control hosts or both mycosed and control hosts (pooled mean ± SEM = 13.08 ± 0.69, $n = 24$ female wasps. Mann–Whitney test: $U = 66.500$; $P = 0.770$). In addition, superparasitism was not observed in either treatment group. However, the distribution of eggs over the 3 d was affected by the sequence of hosts presented to the female wasps (Fig. 7). Numbers of control hosts parasitized on day 1 were similar for both treatment groups but female wasps that did not lay eggs on fungus-treated hosts on day 2, parasitized on average, twice as many control hosts on day 3 than wasps that parasitized control hosts on both days 2 and 3.

Fungus-treated nymphs with no visible hyphae were accepted as hosts by female wasps (Fig. 8). Parasitism was not statistically different between control hosts and fungus-treated hosts incubated for 0 or 24 h (pooled mean ± SEM = 4.34 ± 0.40 parasitized nymphs, $n = 48$ female wasps; Friedman's test: $\chi^2 = 5.519$; df = 3; $P = 0.138$). However, *T. radiata* did not survive on fungus-treated hosts. In the second experiment, no mummification was observed among the 121 nymphs inoculated with blastospores and subsequently parasitized by *T. radiata*. In both experiments, all nymphs dipped in blastospore suspension were dead and partially covered with *Ifr* hyphae within 5–6 d of treatment (Fig. 9a) and completely covered within 12 d (Fig. 9b). In comparison, survival of *T. radiata* was high on parasitized control hosts. Host mummification ($P = 0.443$; Fisher's exact test; two-tailed) and wasp emergence ($P = 0.583$; Fisher's exact test; two-tailed) were similar among parasitized nymphs from the two sets of control hosts in the second experiment. The pooled value for mummification of control hosts was 0.81 (71 mummies from 88 parasitized nymphs) and pooled wasp emergence was 0.74 (51 wasps from 69 mummies because two of the 71 mummies that formed were damaged by handling). Sex ratios of adult wasps from the two sets of control hosts were also similar ($P = 1.000$; Fisher's exact test; two-tailed) and the pooled sex ratio was female biased at 0.37 (19 males among 51 wasps). Recovered female wasps did not exhibit signs of infection by *Ifr* after their exposure to fungus-treated hosts. After 12 d of isolated confinement, none of the 24 female wasps given fungus-treated hosts in either experiment were visibly infected with *Ifr* hyphae before or after their deaths.

Discussion

Introduction and wide-spread establishment of *T. radiata* in Florida and California have resulted in only modest levels of biological control for *D. citri*, with year-round percent parasitism averaging <5% in both states for even groves or urban areas not treated with insecticides (Qureshi and Stansly 2009, Kistner et al. 2016). The presence of *Ifr* was shown to enhance the control of whiteflies and aphids by parasitoids (Mesquita et al. 1997, Avery et al. 2008, Pick et al. 2012). It has likewise been suggested that biological control of *D. citri* could be improved by augmenting *T. radiata* with *Ifr* (Moran et al. 2011, Stauderman et al. 2012, Patt et al. 2015). However, the effective integration of these two natural enemies will require understanding the behavioral and competitive interactions between them.

When a host is attacked by both an entomopathogen and an insect parasitoid, the order and time between infection and parasitism will dictate the interaction and outcome. Development times for fungal entomopathogens are generally shorter than that of parasitoids (Brooks 1993); therefore, prior infection of a host insect by a fungal pathogen will often preclude parasitism (see review by Furlong and Pell 2005). If fungal infection occurs after parasitism, parasitoid survival is usually enhanced by increasing the time between parasitoid oviposition and fungal infection (Powell et al. 1986, Fransen and van Lenteren 1993, 1994; Furlong and Pell 2000). Parasitoids generally deplete their host near the end of larval development, which prevents entomopathogen colonization. Fungal development inside the parasitized insect can also be inhibited if parasitoids release or induce the production of fungistatic substances within the haemocoel of the host over time (Willers et al.1982, Fransen and van Lenteren 1994).

In our study, the same pattern of competitive interaction between *Ifr* and *T. radiata* was observed. Blastospores of our ARSEF

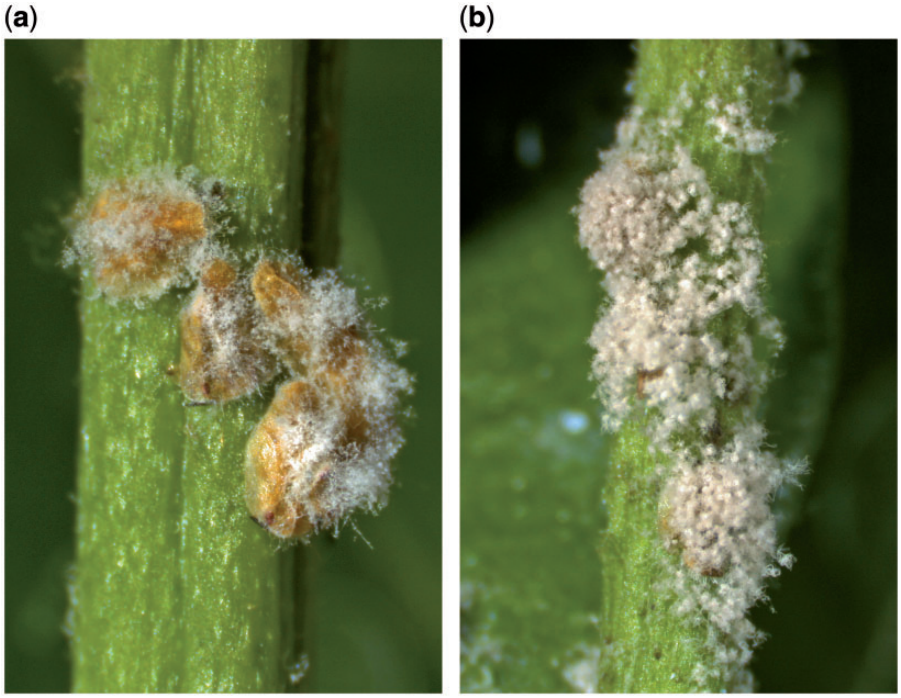

Fig. 9. Clusters of mycosed *D. citri* nymphs that were dipped in *Ifr* blastospore suspensions and incubated for (**a**) 6 d and (**b**) 12 d.

3581 isolate of *Ifr* were capable of infecting *D. citri* nymphs parasitized with second-instar *T. radiata*; the resulting infection greatly reduced host mummification, emergence of adult parasitoids, and the proportion of females among emerged adult parasitoids. In contrast, exposure of fourth-instar *T. radiata* to blastospores had no apparent effects on their survival, emergence, or secondary sex ratio. By the fourth-instar, the *T. radiata* larva had consumed most of the nymph's hemolymph and soft tissue and initiated host mummification. Mummification of the *D. citri* nymph may have played a role in preventing colonization by *Ifr* blastospores. Aphid mummification can induce physical and chemical changes in the host cuticle to prevent infection by entomopathogens (El-Sufty and Führer 1981, St\. Leger 1991). As a result, immature parasitoids residing within aphid mummies are apparently protected from fungal infection (Askary and Brodeur 1999, Kim et al. 2005, Aiuchi et al. 2012). Overall, we found that increasing the time between parasitoid oviposition and blastospore infection decreased the competitive advantage of *Ifr* over *T. radiata*. Similar results have been reported for other cases of intra-host and extra-host competition between entomopathogenic fungi and insect parasitoids (Mesquita and Lacey 2001, Rashki et al. 2009, Martins et al. 2014, Tamayo-Mejí et al. 2016).

Treatments with *Ifr* hyphae negatively affected fourth-instar *T. radiata* in psyllid mummies; however, pupal wasps were not impacted by hyphal treatments. Prepupal *T. radiata* secure the psyllid mummy to the plant surface with silken threads (Chien et al. 1991). In our study, mummies formed 6 d after parasitism and became secured to leaf disks ~7 d after parasitism (Fig. 10a and b). In the hyphal treatments, mummies were entirely surrounded with actively growing hyphae. At 6 d after parasitism, hyphae could have grown under the edge of these mummies and prevented the prepupal *T. radiata* from securing and "sealing" their mummies to the leaf disk, making these immature parasitoids vulnerable to colonization by *Ifr* hyphae or infection by developing conidia. At 9 d after parasitism, mummies containing pupa were sealed and secured to their leaf disks and less exposed to *Ifr* hyphae or conidia. Avery et al. (2010) also found that *Ifr* hyphae from sporulating cadavers of infected adult whiteflies could spread and colonize nearby healthy whitefly hosts. To our knowledge, most published studies on parasitoid susceptibility to fungal entomopathogens have exposed the targeted stages to either blastospores or conidia but not hyphae.

Parasitoids may evade direct competition with entomopathogens by detecting infected hosts and then rejecting them for oviposition (Fransen and van Lenteren 1993, 1994; Lord 2001). Female *T. radiata* did not oviposit on mycosed psyllid nymphs with visible hyphae but did not discriminate against infected nymphs without visible hyphae. Discriminatory behaviors by insect parasitoids toward hosts at advanced stages of infection have been reported for a diverse range of systems (Brobyn et al. 1988, Fransen and van Lenteren 1993, Mesquita and Lacey 2001, Tounou et al. 2003, Rännbäck et al. 2015). Conversely, there are also many reports of parasitoids exhibiting nondiscriminatory behaviors toward infected hosts even though parasitoid survival and fitness are greatly reduced (Aqueel and Leather 2013, Furlong and Pell 2000, Lord 2001, Rännbäck et al. 2015, Tamayo-Mejí et al. 2016).

The contrast in discriminatory behaviors of *Tamarixia triozae* (Barks) (Hympenoptera: Eulophidae) and *T. radiata* toward fungi-infected hosts and healthy hosts stands out because of the biological similarities between the two systems. Tamayo-Mejí et al. (2015) reported parasitism of immature potato psyllids, *Bactericera cockerelli* (Hemiptera: Triozidae), by female *T. triozae* was similar between control nymphs and infected nymphs at 1–6 d following the inoculation of nymphs with *Beauveria bassiana* Bals. (Vuill) (Ascomycetes: Hypocreales). However, they did not report if the infected nymphs showed any visible symptoms of infection that could be detected by female parasitoids. In contrast, we showed that *T. radiata* did not accept mycosed nymphs with visible hyphae.

Some parasitoids may not be able to identify and avoid infected hosts because the interaction is de novo (Lord 2001). In de novo interactions, the parasitoid is unlikely to encounter a fungal entomopathogen,

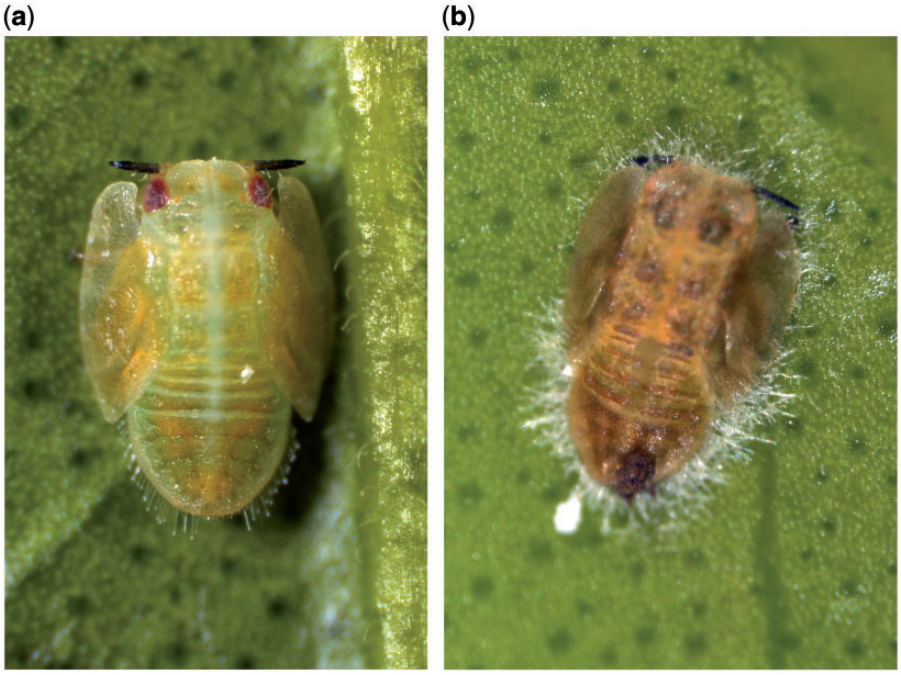

Fig. 10. (**a**) Healthy fifth-instar *D. citri* and (**b**) mummified fifth-instar *D. citri* cemented to a leaf by white threads of silk applied by a fourth-instar (prepupal) *T. radiata*.

so there was no selection pressure to avoid oviposition on infected hosts on which the parasitoid progeny could not survive. There is no information on whether *T. radiata* and *D. citri* may have coevolved with *Ifr* or similar fungal pathogens in their native range. Other species of entomopathogenic fungi are reported to infect *D. citri* in different parts of the world, these include *Hirsutella citriformis* Speare (Ophiocordycipitaceae: Hypocreales) (Meyer et al. 2007), *B. bassiana* (Rivero-Aragon and Grillo-Ravelo 2000), and *Lecanicillium* (formerly *Verticillium*) *lecanii* Zimm (Ascomycota: Hypocreales) (Lu et al. 2015). In Florida, high levels of *D. citri* mortality have been attributed to *H. citriformis* after the rainy season (Hall et al. 2012b). Since *T. radiata* has been widely established in Florida, it would be interesting to determine if the parasitoid avoids psyllid nymphs infected with *H. citriformis*. Comparative studies on interactions among insect parasitoids, entomopathogenic fungi, and shared hosts with coevolved or exotic relationships are needed to better understand discriminatory behavior of parasitoids toward fungi-infected hosts.

Research is needed to identify the external and internal host cues used by *T. radiata* to assess host quality and infection status. Lord (2001) suggested that host recognition by insect parasitoids is often based on the presence of cuticular chemical cues perceived through antennae after contact with the host. Husain and Nath (1923) reported that *T. radiata* females use their antenna to search for suitable hosts while Mann et al. (2010) showed that female *T. radiata* are attracted to the odor of *D. citri* nymphs in a Y-tube olfactometer. Female *T. radiata* also use their ovipositors to extensively probe potential hosts before deciding to either feed or oviposit (Chien et al. 1991). Female parasitoids may use their ovipositors to sense the presence of fungal metabolites in their host's hemolymph (Fransen and van Lenteren 1993, Mesquita and Lacey 2001). It would interesting to know whether visual or chemosensory cues produced by either *Ifr* alone or by infected psyllid nymphs are detected by female *T. radiata* and if the presence of such cues elicits host rejection.

In conclusion, this study found that survival and adult emergence of *T. radiata* were negatively affected when parasitized psyllid nymphs were exposed to *Ifr* blastospores. Although these results are based on laboratory studies, they indicate that *Ifr* could detrimentally affect *T. radiata* if both natural enemies are simultaneously deployed for biological control of *D. citri*. However, immature *T. radiata* in psyllid mummies were not negatively affected by blastospores while hyphae may pose only a limited threat, as when an *Ifr* infection of a *D. citri* population reaches epizootic levels. In addition, female *T. radiata* avoid parasitizing infected nymphs after 48 h of infection. Thus, competition between the two natural enemies could be minimal under field conditions if *T. radiata* females avoid *Ifr*-infected nymphs. Also *Tamarixia radiata* from Pakistan is most active during warmer periods of the growing season for California and Texas citrus, when growing shoots support large numbers of *D. citri* nymphs (Kistner et al. 2016), while ARSEF-3581 and other *Ifr* isolates from the southern United States are most prevalent and infective during the cooler and more humid spring and fall months (Zimmermann 2008). Therefore, we propose that a temporal separation in deployment of the fungus and parasitoid could reduce antagonism and enhance control of *D. citri* in residential landscapes of the LRGV of Texas. Temporal separation of the two natural enemies could be an effective strategy if 1) augmentative releases of *T. radiata* are conducted during late spring to early fall, when peak populations of *D. citri* nymphs coincide with the major flushes of vegetative growth on citrus trees, and 2) deployment of *Ifr* blastospores, via spray or autodisseminators, are reserved for the late fall, winter, and early spring when *D. citri* populations consist primarily of adults. Temporal separation could be an effective and efficient deployment strategy for these two natural enemies of *D. citri* in residential citrus; however, future field evaluations are needed to validate this biological control strategy.

Acknowledgments

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