Chapter 13
Fire Ant Control with Entomopathogens in the USA

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Abstract Fire ants are stinging invasive ants from South America that infest over 129.5 million hectares in the southern United States, where eradication is no longer considered possible. The biological control of fire ants, especially by pathogens, is viewed by some as the only sustainable tactic for suppression. Microscopic-based surveys conducted in South America during the 1970s and 1980s led to the discovery of fungi and microsporidia infecting fire ants. Three of these microorganisms have been studied extensively: *Beauveria bassiana* 447, *Thelohania solenopsae*, and *Vairimorpha invictae*. *B. bassiana* 447 causes fire ant mortality but infections do not spread to queens and intercolony transmission was not evident. *T. solenopsae* has been found in the US and has been shown to spread naturally and debilitate colonies. Colony decline has also been associated with *V. invictae*, which is currently being evaluated for host specificity and possible release in the US. Through the use of molecular techniques, viruses infecting fire ant in the US have been discovered and characterized. *Solenopsis invicta* virus-1 can be transmitted easily to uninfected colonies and colony death often results. This virus apparently causes persistent, asymptomatic infections that actively replicate when the host is stressed. Research on fire ant-specific microsporidia and viruses, as well as other fire ant entomopathogens, is summarized to illustrate the efforts that have been undertaken to understand the biology of these pathogens and to facilitate their utilization in biological control of fire ants.

13.1 Introduction to Fire Ants

Fire ants are stinging invasive ants from South America that plague over 129.5 million hectares in the southern United States (US). "Fire ants" is a name that most commonly refers to *Solenopsis invicta*, which has an official common name in the

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US of "red imported fire ant". In addition to *S. invicta*, the names "fire ant" and "imported fire ant" also refer to *Solenopsis richteri*, the black imported fire ant. These closely related species belong to the *Solenopsis saevissima* species complex and even hybridize where they co-occur. Both species were inadvertently introduced separately into the US (ca 1918 and 1933 for *S. richteri* and *S. invicta*, respectively (Tschinkel 2006)). *S. invicta* is the most prevalent species in the US, mainly occurring in the southeastern states with its northern limits in North Carolina, Tennessee, Arkansas, and Oklahoma. This species is now of worldwide concern with infestations reported from Australia, mainland China, Hong Kong, Taiwan, and Mexico (Tschinkel 2006, Sánchez-Peña *et al*. 2005).

The fire ant inflicts a painful, burning sting and frequently a person will receive numerous stings simultaneously when ants swarm out of their nest to attack an intruder. This greatly intensifies the pain and can cause panic. In addition, it is conservatively estimated that 1% of individuals stung in the US are susceptible to becoming allergic to the venom and at risk for anaphylaxis (Triplett 1976). Deaths from fire ant stings have been reported, and lawsuits have resulted in awards of over $US1 million.

The annual economic impact of fire ants in the US is estimated to be over $US6.5 billion across both urban and agricultural sectors (Pereira *et al*. 2002). In addition, the dominance of fire ants in natural ecosystems has reduced biodiversity and harmed wildlife (Wojcik *et al*. 2001). Given the tremendous impact that fire ants have had in the US, incursions into previously non-infested areas have instigated very expensive eradiation programs. The cost of a planned, but aborted, 10-year eradication program in California was valued at $US65.4 million (Jetter *et al*. 2002). The current eradication program in Australia cost more than $US144 million over 7 years (2001–2007, McNicol 2006).

In the southern US, eradication is no longer considered possible, and instead, integrated pest management (IPM) for fire ants is encouraged. While toxicant-based fire ant baits are the major component of fire ant IPM, the inclusion of parasites and pathogens as biological control agents is increasing. The biological control of fire ants, and especially pathogens, is viewed by some as the only sustainable tactic for suppression of the ubiquitous fire ants. In this chapter we discuss the discovery and use of entomopathogens for fire ant control, from the early surveys utilizing microscopy to the more recent use of molecular techniques to advance microbial control of this notorious invasive pest.

13.2 Past and Present Fire Ant Microbial Control Projects

13.2.1 Natural Enemy/Pathogen Surveys

Classical biological control, that is the release, establishment and spread of effective natural enemies of a pest, is one approach that offers the possibility for permanent regional suppression of fire ants. The arrival of exotic species into new continents

often occurs without introduction of the natural enemies associated with exotics in their areas of endemism. For the red imported fire ant, over 35 natural enemies have been identified in South America (Williams *et al*. 2003) compared to about seven in the US (Collins & Markin 1971, Cook *et al*. 1997, Valles *et al*. 2004). The lack of effective natural enemies can allow exotic species to attain much higher population densities in newly invaded regions than in their native homelands. Accordingly, fire ant populations in the US are generally 5–10 times higher than in South America (Porter *et al*. 1997).

Interest in natural enemies of both *S. invicta* and *S. richteri* extended back into the era when chemical eradication or control using chemicals was being emphasized (Williams *et al*. 2001). In the 1960s Silveira-Guido *et al*. (1973) conducted extensive studies on the parasitic ant *Solenopsis* (*Labauchena*) *daguerrei*, which was found on several *Solenopsis* species in South America. Other parasites, such as *Pseudacteon* phorid flies and *Orasema* eucharitid wasps, have been reported from fire ants. Among these parasitoids, several species of *Pseudacteon* flies have been released as classical biological control agents in the US. Surveys for pathogens of fire ants in South America and the southeastern US have been conducted since the 1970s with several microorganisms eventually being evaluated as biocontrol agents (Williams *et al*. 2003). In 1971 and 1973 surveys of the *Solenopsis saevissima* fire ant complex were conducted in the state of Mato Grosso in Brazil by researchers from the University of Florida. The fungus *Metarhizium anisopliae* and the microsporidium *Thelohania solenopsae* were isolated from *S. invicta* collected in these surveys (Allen & Buren 1974, Knell *et al*. 1977). In contrast to *M. anisopliae*, a cosmopolitan entomopathogenic fungus, the *T. solenopsae* infection was considered to be the first report of a microsporidian infection in ants (Allen & Buren 1974). Subsequent surveys in Uruguay and Argentina in 1974 found *T. solenopsae* in *S. richteri* (Allen & Silveira-Guido 1974). More extensive surveys in Paraguay, Argentina, Uruguay, and Brazil during 1975 and 1976 by researchers from the US Department of Agriculture, the University of Florida, and Ohio State University detected *T. solenopsae* in several other fire ant species in the *S. saevissima* complex (Allen & Knell 1980). Other surveys by USDA researchers, conducted in Brazil in 1976, 1979, and 1981, detected virus-like particles (Avery *et al*. 1977), a neogregarine similar to *Mattesia geminata*, a spore-forming bacterium, and a dimorphic fungus (Jouvenaz *et al*. 1980, 1981). In addition, another microsporidium infecting *S. invicta*, *Vairimorpha invictae*, was discovered and described (Jouvenaz & Ellis 1986), as well as a nematode, *Tetradonema solenopsis* (Nickle & Jouvenaz 1987). Both of these organisms killed infected *S. invicta*, but only *V. invictae* has been evaluated as a biological control agent (see Section 13.2.4). In the mid-1980s, isolates of entomopathogenic fungi from ants collected in Mato Grosso, Brazil were screened for pathogenicity to fire ants. One isolate, *Beauveria bassiana* 447, was selected for laboratory culturing and testing in the US and eventually patented (Stimac & Alves 1994, see Section 13.2.2). In 1987 a project was established at the USDA South American Biological Control Laboratory in Argentina that focused on the ecology of pathogens and other natural enemies of fire ants. This project continues today and has been instrumental in furthering research toward utilization of fire ant pathogens

in the US, as exemplified by an updated pathogen survey and the discovery of a new nematode (Briano *et al*. 2006, Poinar *et al*. 2007).

The search for fire ant (i.e. *S. invicta* and *S. richteri*) pathogens also occurred in the US with limited surveys in 1971 and 1972 in northern Florida and in Mississippi, and a more extensive pathogen survey of fire ants in the southeastern US was completed in 1977. These surveys found only ubiquitous, nonspecific organisms, with the exception of a mildly pathogenic fungus, infecting *S. invicta* (Broome 1974, Jouvenaz *et al*. 1977, 1981). In anticipation of *S. invicta* entering western Texas, a survey of microorganisms in ants in this area was conducted 1978 and 1979, with only one ant from over 2,500 nests being infected with an entomophthoralean fungus, indicating a dearth of ant pathogens in this area (Beckham *et al*. 1982). Interest in finding fungal pathogens continued in 1989 with the screening of colony founding *S. invicta* queens (ca. 1000) collected after a mating flight in Texas. *Metarhizium anisopliae* var. *anisopliae* and a *Conidiobolus* species were isolated from some queens with the *M. anisopliae* isolate exhibiting pathogenicity in a laboratory bioassay (Sánchez-Peña & Thorvilson 1992). Ants collected in fire ant studies in 2000–2003 in Alabama, Florida, and Tennessee resulted in the discoveries of a new *Mattesia* sp. (Pereira et al. 2002, Valles & Pereira 2003a), and the fungi *Myrmicinosporidium durum* (Pereira 2004) and possibly *Akanthomyces* sp. (RM Pereira personal communication) infecting *S. invicta*. *Mattesia* sp. infection, designated as yellow-head disease, was associated with mortality in *S. invicta*, but thus far disease transmission has not been accomplished (Pereira *et al*. 2002).

Molecular biology has provided a new approach for searching for fire ant pathogens. Variants of the intracellular bacteria *Wolbachia* have been confirmed in *S. invicta* populations from the US and South America using *Wolbachia*-specific gene sequences (Shoemaker *et al*. 2003, Bouwma *et al*. 2006). However, the impact of *Wolbachia* on the fitness of *S. invicta* colonies has yet to be documented, with a single variant being examined and its effect insignificant (Bouwma & Shoemaker 2007). Other bacteria have been isolated from *S. invicta* midguts and characterized by 16s rRNA gene analysis and sequencing to potentially provide a vehicle for introducing genes into fire ants for their control (Li *et al*. 2005). The discovery and characterization of SINV-1 and other new viruses in fire ants (Valles *et al*. 2004) also illustrate how molecular techniques can enhance the search for pathogens (see Section 13.3).

Besides the surveys of fire ants to search for potential pathogens, various formulated entomopathogens and entomogenous nematodes originally isolated from other insects have been tested on fire ants. In general, these formulations require direct contact with ants to obtain infection and, in theory, the infection would spread to other colony members and possibly other colonies. However, the removal of cadavers or unhealthy colony members from nests, grooming behavior, antimicrobial secretions, and relocation of nesting sites limits the spread of infections (Oi & Pereira 1993). Applications of nematodes to fire ants resulted in excessive grooming and applications to nests often caused colonies to relocate with minimal colony reductions (Drees *et al*. 1992, Jouvenaz *et al*. 1990).

Bypassing these behaviors should facilitate the spread of an effective pathogen, thus pathogens isolated from naturally infected fire ants conceivably would have more transmission potential. In the following sections we discuss pathogens that have been isolated from *S. invicta* and have had significant assessments of their potential as microbial control agents of this invasive ant.

13.2.2 Beauveria bassiana

Beauveria bassiana is an entomopathogenic fungus that infects many insect species. As mentioned previously, several isolates from *S. invicta* collected in Brazil were screened for efficacy against fire ants in the late 1980s, with one isolate, *B. bassiana* 447 (ATCC 20872; Bb447), being selected for further assessment. This isolate could be cultured efficiently on rice to produce spores (Stimac *et al*. 1993, Stimac & Alves 1994). When *S. invicta* adults were sprayed directly with Bb447 suspensions of 10^8 spores/ml, virtually 100% mortality occurred and evidence of infection was observed in 88% of the cadavers (Pereira *et al*. 1993). However, field application of various formulations of Bb447 spores by injection with pressurized CO_2 into *S. invicta* nests, which are mounds of soil above subterranean networks of tunnels, or scattering rice/fungus on top of nests, resulted in 48–100% of the treated nests remaining active or relocating. Infection was confirmed in 52–60% of adult ants sampled from treated nests and piles of dead infected brood were observed (Oi *et al*. 1994). The lack of control under field conditions could be the result of several factors. Transmission within colonies can be limited by the hygienic behavior of ants, such as the removal of spores by grooming and the discarding of cadavers outside of nests before sporulation (Siebeneicher *et al*. 1992, Oi & Pereira 1993). Pereira *et al*. (1993) demonstrated that mortality of *S. invicta* by Bb447 in non-sterile soil was poor, indicating that soil borne antagonists were hindering infection. Fire ant venom also inhibits the germination of *B. bassiana* conidia (Storey *et al*. 1991). Poor transmission of another isolate of *B. bassiana* spores among fire ants has also been reported (Siebeneicher *et al*. 1992). Pereira *et al*. (1993) conjectured that for transmission to occur, localized pockets of large quantities of spores would need to be deposited within a fire ant nest, and then the pockets must be visited by ants.

In addition to direct spore applications to nests, bait formulations have been developed with Bb447 and other isolates. However, spore contact and germination through the ant cuticle must occur for infection. Formulations that promote such contact, such as attractive, dry powders that are difficult to carry by ants may be most suitable for baits (Pereira & Stimac 1997). Conidia of *B. bassiana* originally isolated from the Mexican leaf-cutting ant, *Atta mexicana*, and subsequently reisolated from *S. invicta* were encapsulated in sodium alginate and then dried into pellets. Alginate, commonly used in processed foods, is a polysaccharide gum extract from algae. These pellets were foraged upon when coated with peanut oil, a food readily accepted by fire ants. The pellets were evidently retained in the nest to allow for sporulation and infection. Significant reductions in fire ant colony activity were reported from field plots where the oil-coated mycelia pellets were broadcast

(Thorvilson *et al*. 2002). However, these results could not be replicated by other researchers (Collins *et al*. 1999, DH Oi unpublished data).

Given the inhibition of *B. bassiana* conidial germination with exposure to venom and soil antagonists and the hygienic behavior of ants, efficiently infecting fire ant queen(s) and the majority of nestmates to kill individual colonies is problematic. In addition, natural intercolony transmission and control have not been demonstrated. Thus, the effective use of *B. bassiana* as a microbial pesticide or a biological control agent against fire ants currently seems unlikely.

13.2.3 Thelohania solenopsae

Thelohania solenopsae is an obligate intracellular microsporidian pathogen that was first observed in alcohol-preserved specimens of *S. invicta* collected in 1973 during a survey in the city of Cuiabá, Mato Grosso, Brazil (Allen & Buren 1974). It is a relatively common fire ant pathogen in South America, being found in 25% of sites surveyed (Briano *et al*. 1995c, 2006). It was noted during the initial survey that infected colonies were smaller and had less vigor when disturbed (Allen & Buren 1974). Subsequent observations in South America suggested that *T. solenopsae* infection was a chronic, debilitative disease causing fire ant populations to decrease rapidly after 1–2 years during periods of stress such as drought (Allen & Knell 1980). Field experiments in Argentina with *S. richteri* documented 83% fewer nests in *T. solenopsae*-infected plots and infected colonies were significantly smaller (Briano *et al*. 1995a,b). The discovery of *T. solenopsae* in the US in 1996 (Williams *et al*. 2003) and the ability to initiate infections by transferring brood from infected colonies (Willams *et al*. 1999, Oi *et al*. 2001) allowed for further documentation of the pathogen's detrimental effects on *S. invicta* colonies in the US. Laboratory inoculations of colonies containing single or multiple (3–12) queens resulted in 88 and 100% reductions in brood within 29 and 52 weeks after inoculation, respectively. Queens from infected colonies weighed less, had declining oviposition rates, and died earlier than queens from healthy colonies (Willams *et al*. 1999, Oi & Williams 2002). Evidence of transovarial transmission was also reported from both *S. richteri* and *S. invicta* (Briano *et al*. 1996, Valles *et al*. 2002). Reductions of *S. invicta* population indices (= estimates of fire ant populations based on the number of ants and the presence of brood in individual nests) in infected field plots in the US were also reported, ranging from "weak" to 63%. Reductions were often due to the presence of smaller colony sizes instead of the total elimination of colonies and reductions often fluctuated (Cook 2002, Oi & Williams 2002, Fuxa *et al*. 2005a). The 6-month or longer decline in colonies most likely allowed for re-infestations to occur, thus documenting field reductions was less consistent (Oi & Williams 2002). Potentiation was exhibited when *T. solenopsae*-infected *S. invicta* died faster after feeding on fire ant bait containing hydramethylnon (Valles & Pereira 2003b), and also after infection by *B. bassiana* (Brinkman & Gardner 2000).

The level of control associated with *T. solenopsae* infection is insufficient especially in urban areas where tolerance to fire ant stings is very low. Perhaps the most

compelling effect of fire ant biological control introductions is the potential delay in re-infestation in areas cleared of fire ants by insecticides. Widespread establishment of *T. solenopsae* and other fire ant biological control agents in unmanaged lands could diminish sources of re-infestations. Infected colonies that split from larger colonies or simply move from untreated to treated areas may eventually die faster. Fire ants can also spread and colonize new areas with newly-mated queens after nuptial flights. *T. solenopsae* was present in fire ant reproductives that initiate nuptial flights as well as in newly-mated queens, which had poorer survivorship and colony founding ability (Oi & Williams 2003). Slower fire ant re-infestation and consistent control was documented in an area where *T. solenopsae* and a fire ant parasitoid, *Pseudacteon tricuspis*, were released and became established (Fig. 13.1, Oi *et al*. 2008). Because the impact of *P. tricuspis* alone is low (Morrison & Porter 2005), *T. solenopsae* may have played the greater role in the slower re-infestation.

S. invicta colonies occur as two social forms: monogyne with one queen per colony and polygyne with multiple queens per colony. Infections of *T. solenopsae* were more prevalent (56–83%) in fire ant populations that consisted of polygyne colonies, and rare (0–2%) in monogyne populations (Oi *et al*. 2004, Milks *et al*. 2008). Polygyne fire ants, including brood and queens, move freely between colonies. Infected individuals can relocate to uninfected nests or healthy ants can move into infected nests, and not all queens within a colony are infected. Thus, infections in polygyne populations can be more persistent (Oi *et al*. 2004). In

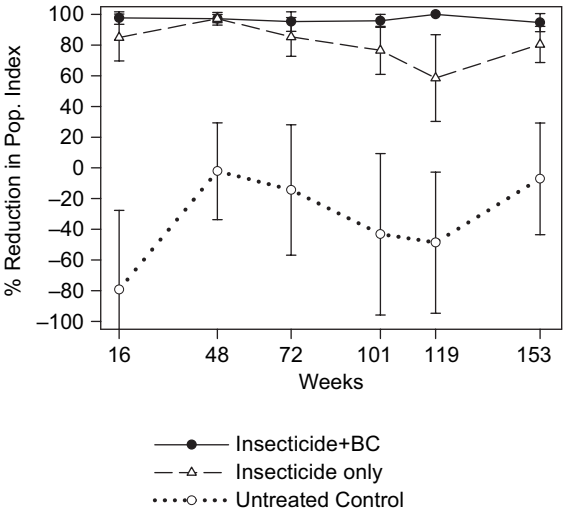

Fig. 13.1 Reductions in *S. invicta* population indices per plot (average % reduction ±95% CI) among areas where (**a**) the fire ant biological control agents *T. solenopsae* and the parasitic fly *Pseudacteon tricuspis* (= BC) were established and insecticide was applied; (**b**) only insecticide was applied; and (**c**) no biological control agents or insecticides were used. Negative reductions represent increases in *S. invicta* populations relative to pretreatment populations. Weeks after insecticide application or biocontrol releases are indicated on the x-axis (adapted from Oi *et al*. 2008)

contrast, monogyne colonies are territorial and there is little intercolony brood or queen exchange. Monogyne colonies infected with *T. solenopsae* apparently succumb without efficient intercolony transmission to maintain or spread the infection. It is also hypothesized that when mixed social forms occur, *T. solenopsae* confers a competitive disadvantage to the social form with higher infection prevalence. In areas of Louisiana where both social forms were living, the social form with more colonies infected with *T. solenopsae* declined more, relative to the other social form (Fuxa *et al*. 2005a, b). In Argentina, *T. solenopsae* infections were found to be nearly equally present in both social forms of *S. invicta* (Valles & Briano 2004). However, the social form assay used may not be applicable to South American fire ant populations and monogyne prevalence needs to be re-evaluated (DD Shoemaker personal communication).

The host range of *T. solenopsae* is apparently restricted to the *Solenopsis saevissima* species group. In South America, besides *S. invicta*, species from which *T. solenopsae* have been reported include *S. richteri* (Allen & Silveira-Guido 1974), *S. saevissima*, *S. quinquecuspis*, *S. macdonaghi*, *S. blumi* [= *S. quinquecuspis*] (Allen & Knell 1980), and *S. interrupta* (Briano *et al*. 2006). Infections in nine other non-*Solenopsis* genera of ants were not detected from samples collected in infected areas in both the US and South America (Williams *et al*. 1998, Briano *et al*. 2002). When infected *S. invicta* brood was introduced to colonies of several ant species from the US, including species from the *Solenopsis geminata* species group (*S. geminata* and *S. xyloni*), infections did not occur. In contrast, introductions of *S. invicta* brood inocula produced infections in the *Saevissima* species group (*S. richteri*, *S. invicta*, and their hybrid) (Table 13.1).

Currently the only known method of consistent intercolony transmission of *T. solenopsae* is by transfer of live, infected brood with rates of up to 80% transmission being reported. In contrast, 4–25% transmission has been reported when brood was tended by infected adult ants (Allen & Knell 1980, Oi *et al*. 2001), with the possibility of contamination mentioned in Oi *et al*. (2001). While four spore types have been described for *T. solenopsae* (Knell *et al*. 1977, Oi *et al*. 2001, Shapiro *et al*. 2003, Sokolova & Fuxa 2001, Sokolova *et al*. 2003, 2004), infection has yet to be initiated by inoculation with isolated spores despite several attempts using spores mixed with various foods (Allen & Knell 1980, Oi *et al*. 2001, Shapiro *et al*. 2003). Chen *et al*. (2004) hypothesized that spores found in the meconia of pupating larvae were the source for horizontal transmission.

Live, infected brood has been used to successfully initiate *T. solenopsae* field infections in several states in the US (Florida, Louisiana, Mississippi, Oklahoma, and South Carolina). These successful inoculations were conducted in polygyne populations of *S. invicta*, with the social form determined by either PCR or by assessment of nest densities in combination with adult worker sizes (Greenberg *et al*. 1985, Macom & Porter 1996). As mentioned previously, sustained infections are most prevalent in polygyne populations, and it is becoming more difficult to find polygyne populations in the US that are completely free of *T. solenopsae*. Establishing sustained infections in monogyne populations has generally been unsuccessful (Fuxa *et al*. 2005a, DH Oi unpublished data). Similarly, since *S. richteri* in the

Table 13.1 Host range of *T. solenopsae* from surveys in South and North America and laboratory inoculations

Field surveys in Argentina and Brazil[a]			
Ant	# Infected nests/ # nests	Ant	# Traps with infected ants/ # traps
Acromyrmex	0/45	*Brachymyrmex*	0/2
Camponotus	0/1	*Camponotus*	0/46
Pheidole	0/4	*Crematogaster*	0/28
S. macdonaghi	11/19	*Dorymyrmex*	0/2
S. invicta	28/255	*Linepithema*	0/10
S. richteri	38/261	*Paratrechina*	0/2
S. quinquecuspis	+[b]	*Pheidole*	0/66
S. saevissima	+[b]	*Wasmannia*	0/1
S. interrupta	+[b]	*Solenopsis* sp.	1/5
		S. invicta	1/67
		S. richteri	22/75
Field survey: Florida, US[c]		**Lab inoculations with US ants**[e]	
Ant	# Infected nests/ # nests	Ant	# Infected colonies/ # colonies
Camponotus floridanus	0/1	*C. floridanus*	0/1
Brachymyrmex depilis	0/1	*Linepithema humile*	0/3
Dorymyrmex bureni	0/9	*Monomorium floricola*	0/3
Pheidole moerens	0/1	*Solenopsis geminata*	0/5
P. metallescens	0/1	*Solenopsis xyloni*	0/8
Trachymyrmex septentrionalis	0/1	*S. richteri* x *S. invicta hybrid*	3/6
Solenopsis geminata	0/15	*S. richteri*	1/7
Pheidole[d]	0/17	*S. invicta*	12/19

[a] Data from Briano *et al*. (2002).
[b] *T. solenopsae* infections reported without quantification (Allen & Knell 1980, Briano *et al*. 2006); + = present.
[c] Data from Williams *et al*. (1998).
[d] Ants collected in Texas, US (Mitchell *et al*. 2006).
[e] DH Oi unpublished data.

US is monogyne, several inoculations of *S. richteri* populations in Mississippi and Tennessee with live infected *S. invicta* brood have failed (DH Oi unpublished data). It was speculated that failure to establish infections in *S. richteri* could be attributed to poor cross-fostering of the *S. invicta* brood despite initially being carried into nests; *S. richteri* being monogyne in the US; and/or host isolate incompatibility.

The method of using live brood as inocula is labor intensive and inefficient. Brood is obtained from field collected *T. solenopsae*-infected colonies and the process of excavating nests, rearing colonies, and separating brood from adult ants is inconsistent, yielding from <1 to $40+$ g brood per colony. Infection rates of inocula per colony are based on the presence of *T. solenopsae* in 10 individual stained slide mounts of fourth instars and/or prepupae, or 10 individual wet mounts of non-melanized pupae. Separated brood (3–5 g) with infection rates of 60–80% is

poured into individual nests where ants from the recipient colony would tend the brood. Shipping or transporting live brood is difficult, requiring careful handling and refrigeration, and inoculating individual nests is time consuming. More efficient methods of inoculation, such as broadcasting formulated inocula, are not available currently. Development of an infective spore formulation(s) that can be stored and is compatible with fire ant bait application equipment would facilitate the dissemination of *T. solenopsae* and perhaps increase infection rates in monogyne populations. Nevertheless, the widespread distribution of *T. solenopsae* in polygyne *S. invicta* in the US and its documented field impact demonstrates its utility as a microbial biological control of fire ants.

13.2.4 Vairimorpha invictae

Vairimorpha invictae is another microsporidium that was described from a *S. invicta* colony collected in the state of Mato Grosso, Brazil, in the early 1980s (Jouvenaz & Ellis 1986, Jouvenaz & Wojcik 1981). *V. invictae* is an obligate, intracellular parasite that produces two spore types: unicellular octospores contained in groups of eight within sporophorous vesicles and binucleate free spores (Jouvenaz & Ellis 1986). *V. invictae* was detected in 2.3% of 2528 *S. invicta* and *S. richteri* colonies at 13% of 154 sites surveyed from 1991–1999 in Argentina. In surveys conducted mainly in Argentina and in portions of Bolivia, Chile, Paraguay, and Brazil from 2001–2005, similar percentages of *V. invictae* infected sites were reported (12% of 262 sites), although the percentage of infected colonies was higher (10% of 2064 colonies). Within individual sites, *V. invictae* prevalence can be high, with 23–83% of colonies being infected (Briano & Williams 2002, Briano *et al*. 2006, Porter *et al*. 2007). In comparison, *T. solenopsae* has been reported to be present in 25% of sites (n = 185 and 262) with 8 and 13% (n = 1836 and 2064, respectively) of colonies infected (Briano *et al*. 1995c, 2006).

Laboratory evidence for the pathogenicity of *V. invictae* included faster mortality among naturally infected, starved adult workers and higher infection rates among dead workers than live workers (Briano & Williams 2002). Infections initiated by the introduction of live infected brood or dead infected adults into small laboratory colonies of *S. invicta* resulted in significant reductions (>80%) in colony growth (Oi *et al*. 2005). Declines in field populations of *S. invicta* (69%) were associated with natural *V. invictae* infections and also simultaneous infections with *T. solenopsae* in Argentina (Briano 2005). The persistence of *V. invictae* field infections appears to be more sporadic with wide and abrupt fluctuations in prevalence, whereas *T. solenopsae* maintains a fluctuating yet sustained infection level (Briano *et al*. 2006). Faster colony declines were observed when simultaneous infections of the two microsporidia occurred in the laboratory (Williams *et al*. 2003).

V. invictae can be found in all life stages of *S. invicta* including eggs. However, the low number of infected eggs and queens makes the importance of vertical transmission in the *V. invictae* life cycle uncertain (Briano & Williams 2002). While laboratory colonies of *S. invicta* can be infected through the introduction of live

infected brood or infected adults that died naturally, infection by isolated spores has not been achieved (Jouvenaz & Ellis 1986, Briano & Williams 2002, Oi *et al*. 2005). However, larvae can be infected when reared to the pupal stage by infected adult workers (Oi *et al*. 2007).

The host range of *V. invictae* appears to be restricted to ants in the *Solenopsis saevissima* species group. Infections have been reported from *S. invicta*, *S. richteri*, and *S. macdonaghi* in field surveys of nests and bait trapping in Argentina and Brazil. In addition, infections were not observed in 10 non-*Solenopsis* genera (Briano *et al*. 2002). Similarly, infections were not detected in 235 non-ant arthropods (10 orders, 43 families, 80 species), and 947 non-*Solenopsis* ants (12 genera, 19 species) collected at baits from five *V. invictae* sites in Argentina (Porter *et al*. 2007). Inoculations with *V. invictae*-infected brood of laboratory colonies of *Solenopsis geminata* and *Solenopsis xyloni*, two fire ant species in the *Solenopsis geminata* species group found in North America, did not result in infections (Oi *et al*. 2007). Thus, the host specificity of *V. invictae* is favorable for release as a biological control agent in the US and efforts are underway to secure approval for its release.

13.3 Molecular Techniques Facilitate Virus Discovery in Fire Ants

Although viruses are considered important biological control agents for use against insect pests (Lacey *et al*. 2001), they have not been examined for their potential use against fire ants. Indeed, before 2004, the only report present in the literature concerned with virus infections in fire ants was the observation of "virus-like particles" in an unidentified *Solenopsis* species from Brazil (Avery *et al*. 1977). As indicated in Section 9.2.1, efforts to discover microbial infections in fire ants in South America were conducted by either brute force examination of large numbers of colonies, or attempts to identify and examine diseased ant colonies, exclusively by microscopic methods (Jouvenaz *et al*. 1977, 1981, Jouvenaz 1983, Wojcik *et al*. 1987). Despite searches over several decades, no virus had been shown to infect *S. invicta*.

In late 2001, a molecular-based approach was employed in an attempt to discover virus infections in fire ant colonies. An expression library was created, sequenced and analyzed to identify potential viral infections through homologous gene identification (Valles *et al*. 2008). The library was created from all stages (eggs, larvae, pupae, workers, and the queen) of a monogyne *S. invicta* colony and 2,304 clones were sequenced. After assembly and removal of mitochondrial and poor quality sequences, 1,054 unique sequences were identified and deposited into the GenBank database (Accession Numbers EH412746 through EH413799). Six ESTs exhibited significant homology with single-stranded RNA viruses (3B4, 3F6, 11F1, 12G12, 14D5, and 24C10). Subsequent analysis of these putative viral ESTs revealed that 3B4 was most likely a ribosomal gene of *S. invicta*, 11F1 was a positive-strand RNA virus contaminant introduced into the colony from the cricket food source (Valles & Chen 2006), 12G12 appeared to be a plant-infecting tenuivirus also introduced into

the colony as a field contaminant, and 3F6, 14D5, and 24C10 were unique and exhibited significant homology (expectation scores $< 10^{-5}$) with the single-stranded Acute Bee Paralysis virus (ABPV) (Valles *et al*. 2004, Valles & Strong 2005).

13.3.1 *Genome Acquisition, Construction, and Characterization of* Solenopsis invicta *Virus-1*

Using these ESTs (3F6, 14D5, and 24C10) as a platform, we conducted 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) to acquire the entire genome of this likely virus. The polyadenylated RNA genome was comprised of 8,026 nucleotides (GenBank Accession Number: AY634314), which encoded two large open reading frames (ORF). These ORFs were flanked and separated by short untranslated regions. The 5′ proximal ORF (defined by nucleotides 28−218 = ORF 1) encoded a predicted amino acid sequence possessing significant identity with the helicase, protease, and RNA-dependent RNA polymerase (RdRp) regions from positive-strand RNA viruses. The predicted amino acid sequence of the 3′ proximal ORF (defined by nucleotides 4390 to 7803 = ORF 2) exhibited similarity to virus structural proteins of positive-strand RNA viruses, especially the Acute Bee Paralysis virus. Electron microscopic examination of negatively stained samples from virus-infected fire ants (as determined by RT-PCR) revealed isometric particles with a diameter of 30–35 nm (Fig. 13.2), also consistent with positive-strand RNA viruses.

This new virus, currently named *Solenopsis invicta* virus-1 (SINV-1), represents the first virus to be discovered in *S. invicta*. SINV-1 was easily transmitted to uninfected *S. invicta* by feeding and the replicative genome strand (or negative strand) was consistently present in infected ants indicating that the virus was replicating and that the ant was serving as host (Valles *et al*. 2004, Hashimoto *et al*. 2007, SM Valles unpublished data). To date, two forms (based on sequence differences) have been de-

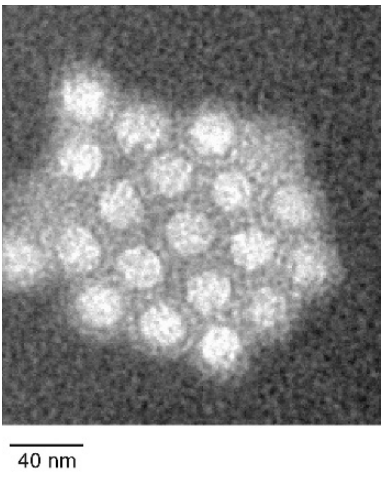

Fig. 13.2 Electron micrograph of *Solenopsis invicta* virus-1 purified from infected fire ants

scribed, namely SINV-1 (Valles *et al*. 2004) and SINV-1A (Valles & Strong 2005). These forms are distinct, and can be differentiated by RT-PCR. However, nucleotide changes result in largely synonymous amino acid changes indicating that SINV-1A is likely a genotype of SINV-1.

SINV-1 is found in all fire ant caste members and developmental stages. Worker ants exhibited the highest genome copy number (2.1×10^9 copies/worker ant) and pupae exhibited the lowest (4.2×10^2 copies/pupa) (Hashimoto *et al*. 2007). Mean genome copy number (based on quantitation of the RdRp) was lowest in early larvae and pupae. Overall, SINV-1 genome copy number increased throughout larval development, declined sharply at pupation, then increased in adults. No symptoms were observed among infected nests in the field. However, under certain situations (stressors), infected colonies exhibited extensive brood death and often colonies collapsed as a result. Thus, SINV-1 fits the paradigm for many insect-infecting positive-strand RNA viruses (Chen & Siede 2007). Specifically, infection is chronic and asymptomatic, which can convert to an active-lethal state under certain conditions. After the initial discovery (Valles *et al*. 2004), research has focused on characterizing and understanding SINV-1 in hopes of utilizing it as a control agent.

13.3.2 SINV-1 Phenology, Host Specificity, Distribution, and Tissue Tropism

Valles *et al*. (2007) reported the phenology, geographic distribution, and host specificity of SINV-1. The prevalence of SINV-1 and -1A among fire ant nests in Florida exhibited a distinct seasonal pattern (Fig. 13.3). Infection rates of SINV-1 and -1A were lowest from December to April, increasing rapidly in May and remaining high through August, before declining again in autumn (October). A significant relationship was observed between mean monthly temperature and SINV-1 ($p < 0.0005,\ r = 0.82$) and SINV-1A ($p < 0.0001,\ r = 0.86$) infection rates in *S. invicta* colonies. Relatively higher temperatures were associated with correspondingly higher intercolony SINV-1 infection rates, a relationship that has been noted previously for other RNA viruses and their insect hosts (Bailey 1967, Plus *et al*. 1975).

SINV-1 was reported to be distributed widely among *S. invicta* populations. It was detected in *S. invicta* from all US states examined (except New Mexico) and Argentina (Valles *et al*. 2007). SINV-1 and -1A were also found to infect other *Solenopsis* species. SINV-1 was detected in *S. richteri* and the *S. invicta/richteri* hybrid from northern Alabama and *S. geminata* from Florida, but not Hawaii. SINV-1A was detected in *S. geminata* and *S. carolinensis* collected in Florida and the *S. invicta/richteri* hybrid from Alabama. However, among nearly 2,000 arthropods collected from pitfall traps from north Florida, none except for *S. invicta* tested positive for SINV-1 or SINV-1A. Thus, SINV-1 appears to be specific to the *Solenopsis* genus, with *S. invicta* and *S. richteri* the main hosts. Positive-strand RNA viruses can exhibit exceptionally wide (e.g. Cricket Paralysis virus) or narrow (e.g. Drosophila

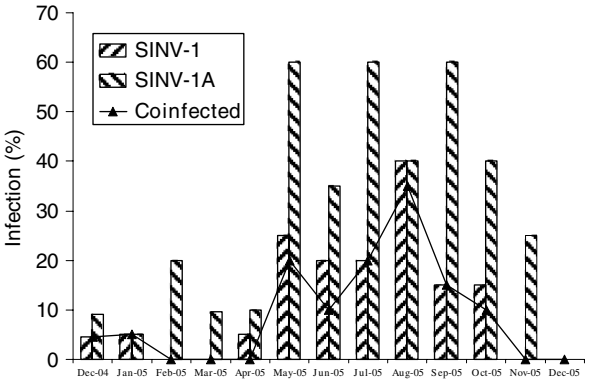

Fig. 13.3 Percentage SINV-1 and SINV-1A infection among field-collected *S. invicta* nests sampled from two locations in Gainesville, Florida. Nests infected with both genotypes are indicated by the line graph. Graph adapted from Valles *et al*. (2007)

C virus complex) host ranges (Christian & Scotti 1998). Although SINV-1 appears to be similar to the Drosophila C virus complex in that it infects only species in a single genus (*Solenopsis*), more direct challenges of other arthropods with SINV-1 in laboratory experiments have not yet been conducted and may indicate otherwise.

Phylogenetic analyses of regions of the SINV-1/-1A genome corresponding to structural proteins indicated significant divergence between viruses infecting North American and South American *S. invicta*. Based on the fact that positive-strand RNA viruses have high mutation rates (Domingo & Holland 1997) in the order of 10^{-4}–10^{-3} per nucleotide site per replication (Holland *et al*. 1982) and recombination does occur via template switching during transcription, the phylogenetic data indicate a significant duration of separation between the virus samples taken from North and South American *S. invicta*. The phylograms also indicate that the North American viral strains have diverged more recently from the common ancestor compared with viral strains from Argentina (i.e. North American strains display fewer nucleotide changes than Argentinean strains). Although hypothetical, these data suggest that SINV-1 was introduced into North America along with founding *S. invicta* or *S. richteri*. This statement is supported by the lack of infection among other *Solenopsis* species (*S. geminata* and *S. xyloni*) in areas apparently devoid (Hawaii) or with incipient infestations of *S. invicta* (Mexico, New Mexico and California). It is further hypothesized that SINV-1 infection of *S. geminata* and *S. carolinensis* (in Florida) may have originated from introduced *S. invicta* and leapt to these native *Solenopsis* species. If this event occurred, it is possible that the SINV-1 infection of native *Solenopsis* species could have provided *S. invicta* with a competitive advantage by reducing the fitness of the native ants relative to *S. invicta*. Further study may provide a more conclusive determination of the origin of SINV-1 and may even provide additional insight into the complex *Solenopsis* phylogenetic relationships. Rapidly-evolving RNA viruses may provide details about host population structure

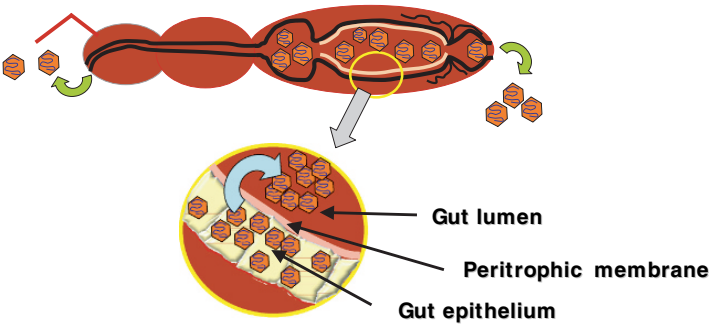

Fig. 13.4 Proposed location of SINV-1 replication and mechanism of transmission. SINV-1 likely replicates in the gut epithelial cells of the midgut. As viral particles are synthesized, they are shed into the gut lumen where they are dispersed to ant nestmates by trophallaxis or substrate contamination by defecation

and demographic history that might not be possible from host genetic data alone (Biek *et al*. 2006).

To determine the susceptible host cell(s), quantitative real-time PCR was employed. This method was also utilized to determine the infection rate among individual ants and colonies of *S. invicta* (Hashimoto & Valles 2007). Among tissues examined from SINV-1-infected ants (larvae and worker ants), the midgut consistently had the highest number of SINV-1 genome copies (>90% of the total). Negative staining and electron microscopy of the supernatant of gut homogenates revealed the presence of spherical virus particles with a diameter of 30–35 nm, consistent with SINV-1 (and positive-strand RNA viruses). Therefore, SINV-1 appears to replicate in gut epithelial cells of *S. invicta*. Viral particles are also found in high abundance in the midgut contents. It is proposed that viral replication occurs in the epithelial cells of the midgut and virus particles are shed into the gut lumen. From there, the particles may be passed to nestmates by trophallaxis or substrate contamination by defecation (Fig. 13.4).

The number of SINV-1 genome copies in infected larvae and workers was often quite high ($> 10^9$ copies/ant). A strong correlation was observed between colony infection rate (number of infected ants/nest) and the number of viral particles (Hashimoto & Valles 2007).

13.3.3 Use of SINV-1 for Controlling S. invicta

Although SINV-1 is currently present in the *S. invicta* US population, we still anticipate its utility as a microbial control agent against this ant pest. SINV-1 appears to fit the paradigm of RNA virus infections in honeybees and other arthropods in that they are present as persistent, asymptomatic infections. However, often when the host experiences certain stressors, the virus begins actively replicating and causes debilitating host symptoms or death. So, there may be a way to emulate these stressors and

induce an active-lethal phase of the viral infection. Furthermore, different strains of SINV-1 are likely to exhibit a range of virulence levels. For example, colony collapse disorder in honeybees has recently been associated with Israeli Acute Paralysis virus of bees (Cox-Foster *et al*. 2007). Investigations on the stress response and conversion to the active-lethal phase of SINV-1 and identification of more virulent genotypes of SINV-1 that could be mass produced for *S. invicta* control are in progress.

13.4 Conclusions and Outlook for Using Pathogens to Control Fire Ants

Over 20 years ago, Jouvenaz (1986) discussed the constraints of using pathogens for the biological control of fire ants. Lacking were knowledge on the biology of fire ant pathogens, such as the mode of intercolony transmission, and efficient methods to screen colonies for pathogens. Examination by microscopy of aqueous extracts has revealed fungi and other spore-forming microorganisms, and now molecular techniques have allowed the discovery and characterization of viruses. Molecular biology will continue to improve our understanding of the biology and ecology of fire ant pathogens and should play a role in solving limitations to pathogen transmission. Constraints such as mass production and dissemination will have to be resolved once the control potential and host specificity of pathogens have been determined. Continuing research on fire ant-specific microsporidia and viruses illustrates the effort to understand their biology and improve their utilization as biological controls for fire ants. Fire ants and their tremendous reproductive capability, mobility, and adaptability, makes their eradication daunting at best. Self-sustaining biological control is one of the few, if not the only, control measure that offers the possibility of long-term regional suppression. Of the known 40+ natural enemies of fire ants, pathogens are a small, but increasing portion as evidenced by the recent discoveries of new viral pathogens infecting *S. invicta* in the US. Additional fire ant pathogens remain to be found, studied, and hopefully introduced from South America. Ongoing research to utilize pathogens for the biological and microbial control of fire ants should eventually yield more measurable benefits and provide a useful model for combating other invasive ants.

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