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Survival of imported fire ant species subjected to freezing and near freezing temperatures

Shannon Suzanne James

To the Graduate Council:

I am submitting herewith a thesis written by Shannon Suzanne James entitled "Survival of imported fire ant species subjected to freezing and near freezing temperatures." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Roberto M. Pereira, Major Professor

We have read this thesis and recommend its acceptance:

Bonnie Ownley, Jerome Grant, Karen Vail

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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And recommend its acceptance:

Karen M. Vail

Bonnie J. Ownley

Jason T. Stratton

Accepted for the Council:

Interim Vice Provost and
Dean of The Graduate School

SURVIVAL OF IMPORTED FIRE ANT SPECIES SUBJECTED TO FREEZING AND NEAR FREEZING TEMPERATURES

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Shannon Suzanne James
May 2001

DEDICATION

This thesis is dedicated to my parents,

Dr. Richard James

and

Mrs. Susan James,

for their constant support.

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ABSTRACT

Originally from the floodplain of the Paraguay River in South America, imported fire ants are well known pests throughout the southern United States. The black imported fire ant, *Solenopsis richteri* Forel, and the red imported fire ant, *Solenopsis invicta* Buren, are believed to have arrived in the United States through Mobile, Alabama, in ships' ballast or dunnage in 1918 and the 1930s, respectively. Current federal quarantine area for fire ants covers portions of 13 states and Puerto Rico including twenty-nine counties in Tennessee.

The top northern portion of the fire ant range (northern Mississippi and Alabama, and southern Tennessee from Shelby Co. to Giles Co.) is inhabited primarily by *S. richteri*. The remaining range is occupied by *S. invicta* with the exception of a sizable band of territory between the parent species that stretches west of the Smoky Mountains to the Mississippi River, which is dominated by their hybrid. A similar pattern of species distribution occurs in their native lands in Argentina, Paraguay, Brazil, and Uruguay.

Two experimental parameters, supercooling point and survival under extended low temperature exposure, were used to examine effects of species and individual size in *S. richteri*, *S. richteri* x *invicta* hybrid, and *S. invicta*, and the effect of *Thelohania solenopsae* (Knell, Allen, and Hazard) infection in *S. invicta* on low temperature survival. Supercooling point is the lowest temperature the insect can be brought to before freezing. Based on supercooling point results for fall-collected ants, *S. richteri* was more cold hardy than the hybrid as shown through a significantly lower supercooling point in large-

and small-sized workers. The spring-collected groups did not show this trend, and instead, the hybrid supercooled lower in the large-sized ants and there was no significant difference in the small-sized ants. Winter-collected large and small *S. invicta* infected with *T. solenopsae* supercooled to lower temperatures than those not infected. However, spring-collected colonies gave the opposite result. Large spring-collected *S. richteri* workers had supercooling points not significantly different from the *S. invicta,* but the hybrid had a significantly lower supercooling point than the parent species. Small hybrid ants were not significantly different from *S. richteri*, but the *S. invicta* supercooled to a significantly lower temperature.

In the extended exposure tests, the chilling injury of imported fire ants was measured through monitoring ant mortality during 15 days of exposure to one of three temperature regimes: +4°C, +0.5°C, and -4°C. Under the +4°C regime, both the hybrid and *S. invicta* infected with *T. solenopsae* had significantly lower mortality rates than either the *S. richteri* or the uninfected *S. invicta* by day seven. The +0.5°C regime caused increased ant mortality when compared to the +4°C regime, and the hybrids had significantly lower mortality than uninfected *S. invicta* from day five through the last day of exposure. The *S. richteri* and infected *S. invicta* were not significantly different. One hundred percent mortality was reached within all groups by day seven in the negative temperature regime. The uninfected *S. invicta* was consistently less cold tolerant than the other groups. At all three regimes, the uninfected *S. invicta* had the highest mortality and the hybrid had the lowest of all groups.

The supercooling point does not seem to be an appropriate measure of cold hardiness in imported fire ants. It is too easily affected by outside phenomena, such as time in the lab and thermocouple size. The differences among the supercooling points of different ant groups were not consistent and these significant differences slight, in light of natural cold weather phenomena. Furthermore, results from the extended exposure tests displayed fire ant mortality at temperatures well above their supercooling points. These results support the hypothesis that extended cold injury causes winterkill of fire ants, and may partially explain the species distribution of fire ants.

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A. Imported Fire Ants

i. History of United States Invasion and Range

Originally from the floodplain of the Paraguay River in South America, imported fire ants are well known pests throughout the southern United States. The black imported fire ant, *Solenopsis richteri* Forel (Hymenoptera: Formicidae), and the red imported fire ant, *S. invicta* Buren, arrived in the United States through Mobile, Alabama, in ships' ballast or dunnage in 1918 and the 1930s, respectively (Jemal and Hugh-Jones 1993). They rapidly spread from the Mobile area, infesting 252,610 km^2 by 1958 and 1,140,459 km^2 by 1995 (Callcott and Collins 1996). Current federal quarantine area for fire ants covers more than 1,253,989 km^2 (A. M. A. Callcott pers. comm.) throughout 13 states and Puerto Rico (Fig. 1.1) (Code of Federal Regulations 2000). Tennessee has 29 counties along the state's southern border included in the federal quarantine range (Fig. 1.2).

General species segregation occurs within the total imported fire ant range (Fig. 1.3). *S. richteri* can be found in the northern portion of the fire ant range in the United States (Callcott and Collins 1996), an area approximately from 34.5 to 35.5 °N latitude (Shoemaker et al. 1996, Vander Meer unpublished data, Milam pers. comm.) that covers parts of northern Mississippi and Alabama, and southern Tennessee from Shelby

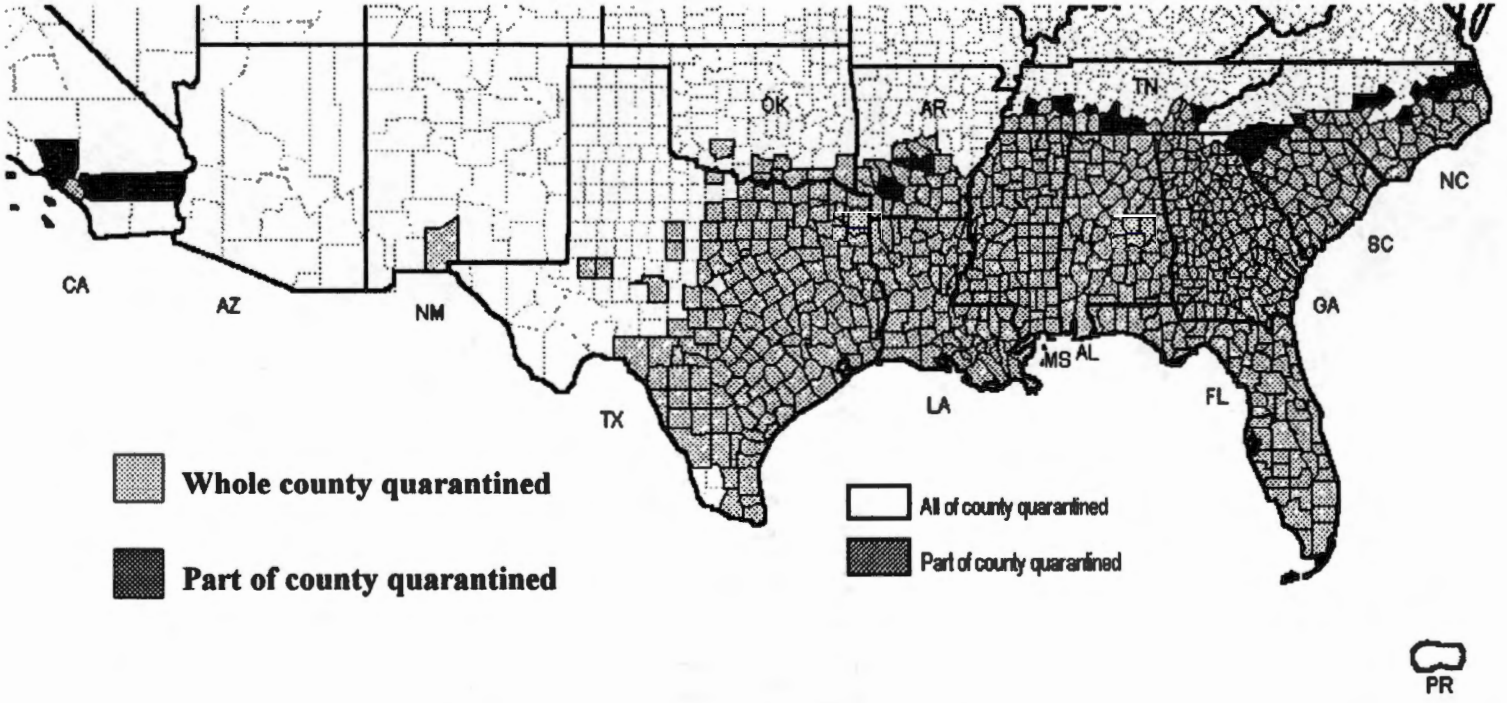
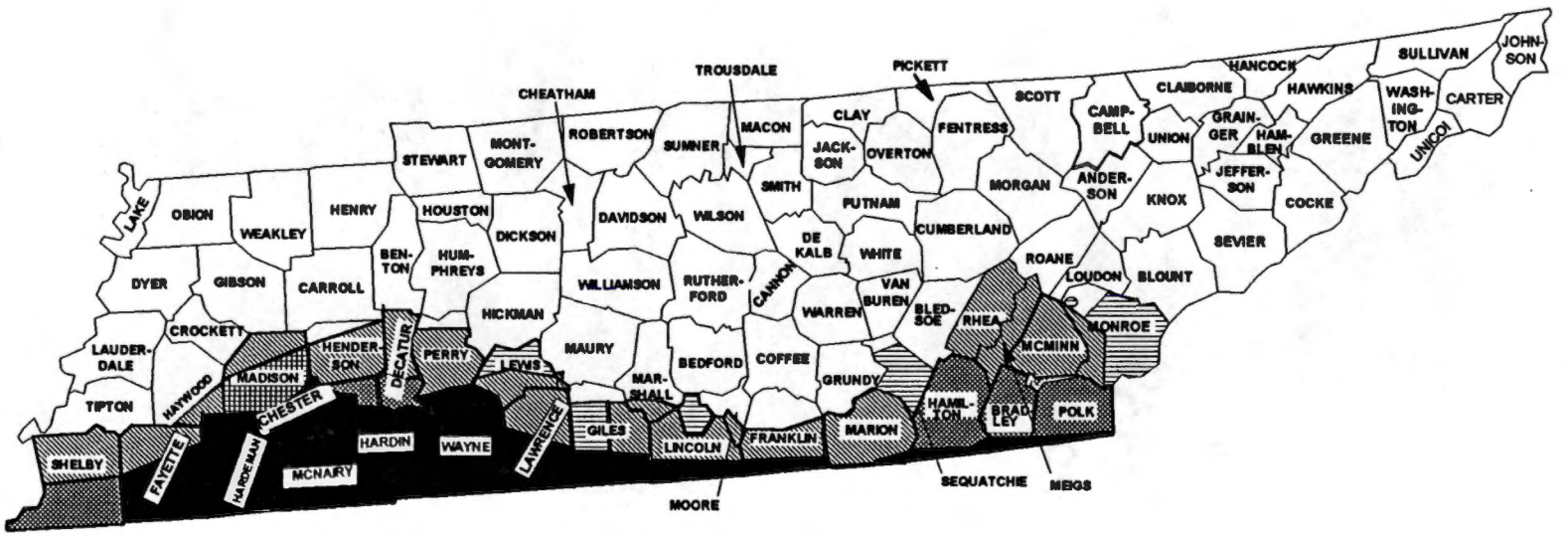


Figure 1.1. Imported fire ant quarantine zone in the United States in 2000 showing counties either partially or entirely under quarantine (U.S. Department of Agriculture – Animal and Plant Health Inspection Service 2000, http://www.aphis.usda.gov/ppq/maps/fireantjpg).



Regulated 1996 Regulated 1997 Regulated 1998

Regulated 1999 Regulated 2000

Figure 1.2. Imported fire ant quarantine zone in Tennessee, showing progression from 1996 to 2000 (Modified from Tennessee Department of Agriculture Imported Fire Ant Quarantine Map for 2000 provided by Gray Haun of the Tennessee Department of Agriculture).

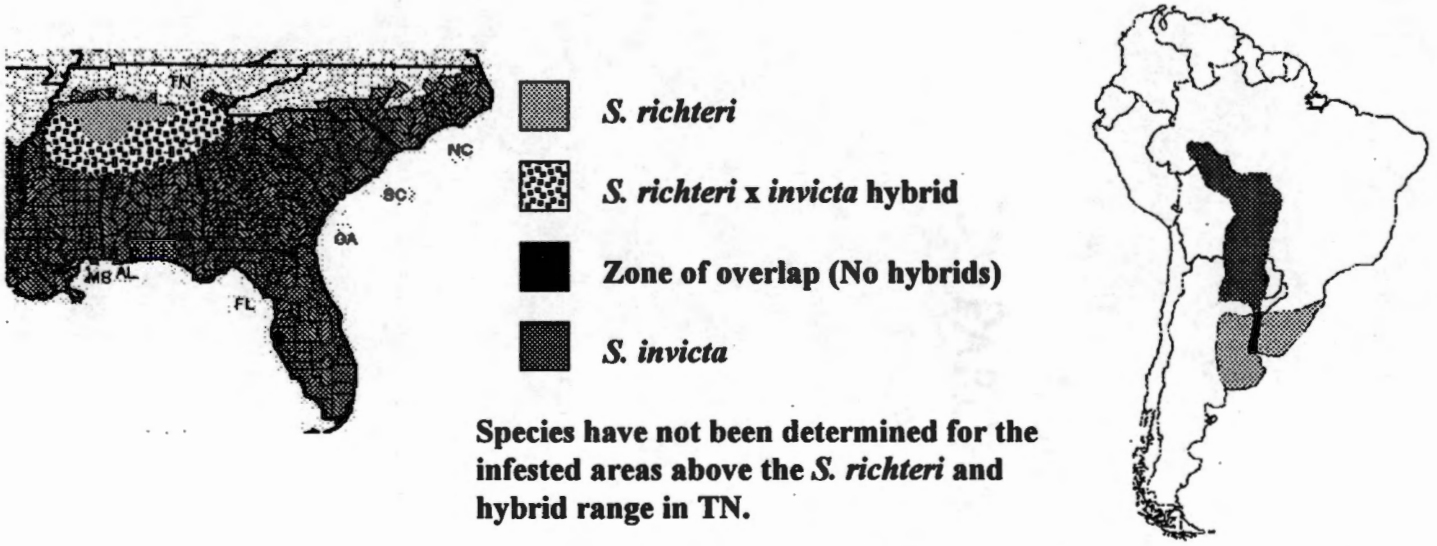


Figure 1.3. Geographic distribution for *S. richteri*, *S. richteri* x *invicta* hybrid, and *S. invicta* in their introduced (North American) (modified Figure 1.1 according to Callcott and Collins 1996, Drees et al. 1998, Shoemaker et al. 1996) and native (South American) ranges (Stimac and Alves 1994).

Co. to Giles Co. The South American range for *S. richteri* is in Brazil, Uruguay, and Argentina from about 30 to 40°S latitude (Buren et al. 1974, Stimac and Alves 1994). *S. invicta* occurs throughout the southern portion of the United States and is the sole infesting species of imported fire ant in all quarantined states except Mississippi, Alabama, Georgia, and Tennessee. The *S. invicta* of South America are found approximately from 8 to 26°S latitude in portions of Brazil, Bolivia, Paraguay, and Argentina and extend slightly into *S. richteri* territory along the Paraguay River system (Buren et al. 1974, Stimac and Alves 1994). The *Solenopsis* hybrid, which is not found in South America, cuts a sizable band of territory east of the Mississippi between the parent species (Callcott and Collins 1996, Drees et al. 1998, Shoemaker et al. 1996).

ii. Impacts of Imported Fire Ant Infestation

The aggressive fire ants possess a painful sting and cause adverse effects in agriculture, wildlife, and urban settings. Agricultural losses due to imported fire ant infestations occur on several levels from direct damage to crops, animals, and equipment, to the cost of control, and lost time dealing with the fire ant problem and its consequences (Drees et al. 1998). Livestock in infested areas are frequently stung and may be kept from food and water because of fire ant presence. Eyes, nose, mouth and other openings on newborn, incapacitated, or low-to-the-ground animals, as well as hatching eggs, are particularly attractive to the ants (Sorensen 1988). Fire ants have been documented attacking numerous crops in various stages of development (Lofgren 1986a) causing considerable loss in soybean (Adams et al. 1983), citrus (Banks et al. 1991), sunflower

(Stewart and Vinson 1991), okra, and corn (Lofgren 1986a). The presence of the ant mounds in the field causes problems as contact with mounds may damage equipment or block it from effective use (Adams et al. 1977). Fire ant attacks on agricultural workers can cause serious problems (Banks et al 1991). Imported fire ants also aid in the spread of homopteran-carried diseases as they tend, protect from predators, and help relocate aphids (Michaud and Browning 1999, Vinson and Scarborough 1989). Quarantine legislation does not restrict movement of equipment and goods within the quarantine zone, but shipments from quarantine areas to non-quarantine areas of hay, nursery products, soil, and any other products or equipment kept in contact with soil must be certified or go through inspection and deinfestation, causing added expenditures (Code of Federal Regulations 2000).

Imported fire ants reduce biodiversity in ecosystems through competition with and predation on native fauna. Fire ant attacks on deer (Allen et al. 1997) and ground-nesting birds, such as quail (Allen et al. 2000), have been documented. There are also reports of general decreases in populations of certain vertebrates in the presence of fire ants and corresponding increases in vertebrate diversity and abundance occurred where fire ants have been controlled (Allen et al. 1997, Trostle 1988). Direct competition with native ants resulting in decreased diversity in ant assemblages is to be expected (Camilo and Philips 1990, Wilson and Brown 1958). Vinson (1991) reported on the decimation of the decomposer arthropod community on vegetable matter, as the imported fire ants not only competed for the same food resources, but also attacked adult foragers and ate the decomposer eggs and larvae.

An estimated 11 to 14 million people are stung annually in the United States. Most stings will only result in minor irritation and a small pustule at the sting location, but allergic reactions do occur and sometimes lead to death (Jemal and Hugh-Jones 1993). The opportunistic fire ants readily colonize sunny, open, disturbed areas, such as lawns and roadsides (Tschinkel 1986), bringing the ants into urban developments and in contact with humans. They will invade buildings (Bruce et al. 1978), and recently, bed-ridden elderly people have died from attacks (Cramer 2000, Associated Press 1998). Further inconvenience is caused through the fire ant habit of building nests in, and shorting out, electrical equipment, such as air conditioning units, telephone boxes, and traffic signals (MacKay et al. 1991, Vinson and MacKay 1990, Lofgren 1986a).

As insect predators, imported fire ants have occasionally been reported as beneficial insects. The sugar cane borer, *Diatraea saccharallis* F. (Lepidoptera: Pyralidae), responsible for most insect damage in sugar cane crops, increased in numbers in Louisiana when fields were treated to control fire ants (Long et al. 1958 in Lofgren 1986a). Brinkley et al. (1991) saw no significant loss in Texas cotton production when fire ants were left to control the four top pests instead of using chemical treatments. A three-fold decrease of fall armyworm, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), in sweet sorghum also has been attributed to fire ant predation (Fuller et al. 1997). Fire ants have contributed to significant population reduction in ticks (Fleetwood et al. 1984) and mosquitoes (Lee et al. 1994), an action that could be considered helpful to human health (Jemal and Hugh-Jones 1993). Unfortunately, most research on imported fire ant

interactions fail to take into account both the beneficial and pestiferous results, and instead focus only on one or the other effect.

iii. Identification

Imported fire ant workers may be identified by ten-segmented antennae terminating in a two-segmented club, an unarmed propodeum, a two-segmented waist, a bicarinate clypeus that produces a tooth at the anterior end of each carina, a mostly smooth, shining integument with many erect hairs of differing lengths, and their sting. Female sexuals have ocelli, large eyes, 11-segmented antennae, which like those of workers, also ends in a two-segmented club, and the same coloration as workers. The males have much smaller heads than the female sexuals, are black with the exception of pale antennae, have large eyes and ocelli, and lack carinae (Buren 1972).

The coloration of *S. richteri* is black to dark brown and many larger workers and the female sexuals have an orange to yellow tergal spot with a well-defined border on the gaster. *S. invicta* usually is lighter and without the tergal spot, or, if present, the spot is indistinct (Trager 1991, Buren 1972). The *S. richteri* x *invicta* hybrid often looks more like *S. richteri,* but with faded coloration and with a less distinct tergal spot. However, the hybrid may look like either parent species (Trager 1991, Vander Meer et al. 1985). Therefore, fire ants from regions where multiple species exist should be tested through methods other than morphology, such as allozyme, RAPD DNA, or gas-chromatograph analysis for accurate species determination (Shoemaker et al. 1996, Ross et al. 1987).

iv. Ecology and Biology

Imported fire ants, regardless of species, share certain biological and behavioral characteristics. Mature colonies may consist of one or more fertilized queens, alate male and female sexuals, sterile female workers, and brood. Eggs, any of the four larval instars, and pupae are collectively termed brood (Vinson and Greenberg 1986). Newly-mated queens that find a suitable site excavate a chamber in which to lay the first clutch of 10-20 eggs. Approximately 30 days later, the new workers emerge, and the queen starts producing 75 to 200 eggs daily (Markin et al. 1973). In about two to three years a colony reaches maturity, containing 100,000 or more workers (Markin and Diller 1971).

Workers switch tasks as they age. Usually young workers start off tending the brood, followed by food storage, nest maintenance, colony defense, and finally, moving out of the nest to forage. Smaller workers often tend the brood and the queen, while larger workers are utilized more in acquisition of large food particles (Tschinkel 1986). Adult fire ants consume only liquids; the fourth-instar larvae are the only members of the colony that can consume solids. Fourth-instar larvae process solid foods and then regurgitate the digested food, which is fed to the rest of colony (Petralia and Vinson 1978). Fire ants do not hibernate, but do decrease activity in cold weather, and brood production slows or stops depending on environmental severity (Francke and Cokendolpher 1986).

Mating flights usually occur in the spring and fall, but may occur any season if the temperature is between 24 and 33°C, the wind is low, and relative humidity is 80% or higher. Usually after rain under these conditions, the workers will make numerous openings in the mound and alates may be seen walking on the surface preparing for the

nuptial flight. The flight starts with the males leaving in the late morning to early afternoon and hovering at an altitude of about 60 to 150 m. The females leave about 30 min after the males and fly through the cloud of males, mate, land, remove their wings, and start a new colony (Markin et al. 1971). Fire ant colonies can contain one queen (monogyne) or many queens (polygyne). In monogyne colonies, unless the colony is queenless, workers usually will kill newly mated queens that land near their fire ant mound (Vinson and Greenberg 1986). Unmated females remaining in the nest may dealate and produce unfertilized eggs, from which only males emerge (Voss 1981).

Fire ants naturally disperse by mating flights, but whole colonies can be moved through floating on floodwaters. Sometimes dispersal is unintentionally aided by man as newly mated queens, small colonies, or parts of colonies go unnoticed in or on equipment, nursery products, hay, or vehicles. Ninety percent of the new infestations from 1945 to 1955 were associated with infested nursery products (Hung and Vinson 1978).

Polygyne colonies have been commonly found among *S. invicta* in Texas and parts of Florida, Mississippi, and Georgia (Bhatkar 1990). Mounds of polygyne colonies are usually much smaller than those of monogyne colonies (Macom and Porter 1996). Polygyne queens are less fecund than monogyne queens (Vargo 1990), produce fewer sexual brood (Vargo and Fletcher 1987), and the workers are usually smaller (Greenberg et al. 1992). The polygyne colonies are less aggressive towards one another, thus permitting increased colony densities and interconnected supercolonies. This increase in density, to approximately three times that of monogyne colonies, along with higher worker numbers per colony, give polygyne infested areas an average of 4,100 ants/m^2 as opposed

to 2,200 ant/m^2 found in areas with monogyne colonies (Macom and Porter 1996).

In their native South American range, fire ants do not populate areas as densely as in their North American territory and thus are not usually considered pests (Briano et al. 1995). Ant sampling along roadsides in South America yielded only 23% of the baits with fire ants, while in North America, 79% of the baits were occupied by fire ants. Even polygyne sites in South America have lower ant density than those in North America (Porter et al. 1997). This difference in population is attributed to the introduction of fire ants into the United States without many of their natural enemies (Briano et al. 1995, Porter et al. 1997).

Fire ant colonies are most recognizable when associated with the typical mature fire ant mound. When disturbed, thousands of ants immediately pour out of the nest to defend it. Above ground, mounds typically appear as a dome-shaped soil structure, usually without holes in its surface. Mature mounds reach dimensions averaging approximately 40 cm high and 50 cm wide, but soil type, structures, and vegetation influence the shape and texture of the mound (Green 1967). The average monogyne nest volume is 14.7 L in sandy soil (Macom and Porter 1996). Underground chambers and tunnels extend several meters into the soil to reach moisture. Foraging tunnels may radiate several meters from the mound just under the soil surface. It is through openings in these foraging tunnels that the ants enter and exit the nest (Vinson and Greenberg 1986). Nests are often located in open sunny areas, such as pastures, lawns, and fields, but may be in buildings or associated with other structures that provide shelter (Hubbard and Cunningham 1977, Wojcik 1983)

v. Control

Fire ant control relies upon the death of the colony's queen(s), so toxins that act too fast provide poor long-term control because they kill workers and never reach the queen(s) (Drees et al. 2000). The weed-like biology (rapid reproduction and easy dispersal) allows rapid fire ant reinfestation of treated areas (Tschinkel 1986), so environmentally friendly treatments with a low residual time may not be effective (Drees et al. 2000). These situations make effective control options for imported fire ants hard to find and leave people in uninfested areas to rely heavily on the quarantine efforts to limit fire ant spread.

Chemical insecticides have been used to control imported fire ant populations since calcium cyanide dust was used in 1937. Chlorinated hydrocarbons, such as chlordane, heptachlor, and dieldrin, were used as residual and contact controls for fire ants in large-scale applications during the early years of the fire ant control program initiated by the United States Congress in 1957. Use of these chemicals was later prohibited due to their effects on the environment. Mirex, a bait-formulated toxin, which was easy to apply and reduced exposure to non-target wildlife, replaced the contact poisons. However, non-target organisms, including humans, showed mirex, chlorinated hydrocarbon, residues regardless of bait formulation. Mirex use was thus cancelled in 1978 (Lofgren 1986a, Lofgren 1986b, Williams 1994).

Currently, chemical controls for fire ants fall into two major categories contact poisons and baits. Contact poisons rapidly reduce numbers of ants in treated colonies and are easy to use for smaller areas of infestation, but they can miss the queen and foraging

workers thus allowing the colony to recover. Most baits operate as either metabolic inhibitors or insect growth regulators. Baits are more likely to affect the queen thus killing the whole colony, are less labor intensive for large areas, and give longer suppression than contact insecticides (Lofgren 1986b, Williams 1994). However, control may not be observable for about one month after application (Williams 1994). Baiting may have negative or positive impacts on native ants that compete with fire ants. Fire ants, with their higher reproductive rate, often repopulate and dominate the treated area before other ants (Tschinkel 1986, Williams 1986).

Research is currently being conducted on many organisms that are thought to reduce fire ant populations within their native range. *Thelohania solenopsae* Knell, Allen, and Hazard (Microsporidia: Thelohaniidae) has shown much promise as a biological control agent. Studies have demonstrated *T. solenopsae* as a widely distributed fire ant-specific pathogen that is often the sole natural enemy causing significant reduction in fire ant populations (Briano et al. 1995). This pathogen spreads easily as it can be transmitted from an infected queen to her eggs and from ant to ant. *T. solenopsae* seems to reduce brood production and shorten life span of the queen, but effects on other castes of infected fire ants are as yet unknown (Williams et al. 1999). It is hoped that establishment of fire ant specific-biological controls, such as *T. solenopsae,* will reduce the fire ant population so that native ants may effectively compete and thus decrease fire ant populations even further (Jouvenaz 1986, Briano et al. 1995).

Environmental extremes detrimental to imported fire ant survival are being studied for use in imported fire ant control. Regions to the west of the current fire ant range are

supposedly too arid to support these ants (Cokendolpher and Francke 1985), though desiccation resistance has been recently documented in west Texas populations of *S. invicta* (Phillips et al. 1996). Areas to the north are expected to have winters too cold for survival of fire ants (Francke and Cokendolpher 1986). Ice-nucleating bacteria have been tested as a possible control measure during cold weather for freeze-intolerant insects (Fields 1993). Ice forms slowly and at lower temperatures when the initial crystallization relies upon pure water, but ice-nucleating bacteria provide an efficient template for crystallization, thus allowing freezing to occur at warmer, sub-zero temperatures (Lee and Costanzo 1998). Experiments using the ice-nucleating active bacterium *Pseudomonas syringae* Van Hall (Pseudomonadales: Pseudomonadaceae) on red imported fire ants have shown an increase in the freezing point (Landry and Phillips 1996). The imported fire ant is not freeze-tolerant, thus, once frozen these ants are dead. This potential of *P. syringae* to increase winter-kill of imported fire ants may be important in the northern areas of the fire ant range, such as southern Tennessee.

B. Effects of Low Temperatures on Insects

i. Cold Injury

Low temperatures cause a number of injurious situations for many insects. Three main categories of cold injury are bodily freezing, direct cold injury (also called cold shock), and indirect chilling injury. An insect is considered frozen when its hemolymph undergoes crystallization due to low temperatures. Insects not freeze-tolerant die after

freezing. Both direct and indirect cold injuries occur at temperatures well above the freezing point of the insect's bodily fluids (Lee 1991, Kelty et al. 1996). Insects may experience death, damage not immediately lethal, or an impairment of function from undergoing direct or indirect cold injury (Lee 1991).

a. Freeze Damage

Investigations performed on microbial and mammalian cells have provided some insight into possible mechanisms of lethal or impairing damage to insects involved in cold and freezing situations. During ice formation, water separation from other hemolymph components may lead to osmotic loss of cellular water causing cellular dehydration (Mazur 1984, Karow 1991) and the denaturing of proteins due to a subsequent altered pH (Denlinger and Lee 1998). Another hypothesis considers the existence of a critical minimum cell volume that once surpassed, due to osmotic water loss, causes damages from which the cell cannot recover from when water returns to the cell after thawing (Meryman 1974). Lysing of cells is also a possible source of damage, because the cytoplasm, while under freeze conditions, is hypertonic and could cause a rapid influx of water upon thawing (Denlinger and Lee 1998). Ice formation and reformation may cause mechanical damage to cells and tissues (Luyet 1966).

b. Non-Freezing Cold Injury

Direct cold injury is due to a rapid rate of temperature drop usually with brief exposure that may lead to cellular membrane damage (Denlinger et al. 1991). Indirect cold injury results from long-term exposure to temperatures near 0°C, but the mechanism(s) of damage remain undetermined (Lee 1991). Irreversible injury may occur due to decrease in

enzyme activity, protein denaturation, and phase transitions in the plasma membrane. Phase changes in the plasma membrane could alter permeability and activity of membrane bound enzymes. After a membrane exposed to low temperatures enters gel phase, it may have permanent segregation of its lipids and proteins (Quinn 1985, Hazel 1995). Direct cold injury also may be attributed to thermoelastic stress from the cell membrane condensing beyond its capacity to hold cellular contents (McGrath 1984). Another theory suggests free-radical injury to the cell may occur from cold-damaged mitochondria and electron transport proteins, as some cold-hardy varieties of plants show increased levels of free-radical-binding compounds (Walker and McKersie 1993, Roxas et al. 1997).

The neuromuscular system of insects seems to display cold-exposure injury first (Denlinger and Lee 1998). Cold coma, usually the first visible sign of cold effects, occurs when the electrical excitability from muscles and nerves is lost, leading to the inability of the insect to move. As temperatures drop, resting and muscle action potentials decrease while duration of muscle potential increases, thus resulting in gradual slowing of movement (Hosler et al. 2000). Cold coma is a reversible, non-injurious situation if exposure is not prolonged (Goller and Esch 1990, Hosler et al. 2000). Irreversible damage to the neuromuscular system frequently results from extensive cold exposure. Experiments exposing *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) to detrimental but not immediately lethal low temperatures rendered them unable to perform regular tasks like grooming, impaired response to stimuli (Kelty et al. 1996), and caused inability to properly develop into functional adults (Yocum et al. 1994).

Reproductive ability and tissue development may be damaged during susceptible periods of an insect's development (Sehnal 1991). Female *Musca domestica* L. (Diptera: Muscidae) had a shortened life span and produced fewer eggs daily after direct cold injury during the pupal stage. Additionally, eggs that were produced had a low emergence rate (Coulson and Bale 1992). *Galleria mellonella* L. (Lepidoptera: Pyralidae) experience solidified silk proteins and alteration of silk glands when non-lethal cold injury occurs. Cold injury to some beetles, such as *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), may cause a release of juvenile hormone leading to retention of pupal features after ecdysis (Sehnal 1991).

ii. Cold Injury Avoidance

a. Behavioral Tactics

Regardless of whether an insect has the physiological means of avoiding cold injury, winter survival strategies are usually initiated through behavior. Few insects migrate such as the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Danaidae), but many seek some protection from harsh conditions. Often the cues used to trigger diapause or quiescence, such as decrease in temperature, change in photoperiod, senescing of host plant (Danks 1991), or possibly a reduction in food availability (Ito and Noor 1993), induce the search for or construction of winter shelter. Often, species move from the summer habitat to a different wintering one. Many crop pests move from the field to protective hedgerows or woods (Slosser and Boring 1980, Burgess 1981, Fye 1982). Boxelder bugs, *Boisea trivittata* Say (Hemiptera: Rhopalidae), and the multicolored Asian

lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), are well known for entering buildings to escape winter conditions (Williams 1995, Vail 1998). Others may move deeper into the substrate where they are already located, as in the case of leaf-litter or soil-dwelling insects (Smith and Flessel 1968, Kirk 1974), and some plant pests like the southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae), that overwinter in plant stems.

Overwintering site selection is dependent on the needs of the organism. Insects overwintering close to or in open air conditions are at greater cold-exposure risk, but will be able to take advantage of warm weather faster than those overwintering in more protected locations (Danks 1991). Aggregation of some insects at an overwintering site may provide advantage in mating when warm weather returns (Hagen 1962), protection from predators, and a buffer against temperature change (Benton and Crump 1979). Most hibernacula provide moisture-related protection. Desiccation resistance, resistance to inoculative freezing, and buffering against rapid temperature drop are, at least in part, determined by the moisture levels present in the hibernacula and are key in winter survival (Danks 1991, Costanzo et al. 1997). Avoidance of inoculative freezing is important to most insects, as contact with external ice initiates ice formation within the insect at temperatures well above their normal freezing temperature (Frisbie and Lee 1997). Evacuation of gut contents is a tactic used to further avoid ice formation inside the insect (Cannon and Block 1988).

b. Physiological Tactics or Cold Hardening

The winter-active insects, those active at low winter temperatures, have physiologies different from most temperate zone insects. While winter-active insects have special adaptations that allow them enzymatic and physical activity at low temperatures, they may not display true cold-hardening as they may not go through change in cold-tolerance level (Heinrich 1987). Many temperate-zone insects, however, undergo one or more physiological changes regardless of their winter level of activity. The three main categories of physiologically-based, cold-injury avoidance tactics are: recovery from freezing (freeze tolerance), avoidance of ice formation in bodily fluids (supercooling), and a lessening of damage caused by exposure to cold, non-freezing temperatures (acclimation) (Lee 1991).

1. Freeze Tolerance

Freeze-tolerant insects can use a number of techniques to increase their supercooling point. By freezing at higher temperatures, these insects protect their tissues from osmotic shock and have slow ice crystal formation. This extracellular process usually takes hours or days to complete causing little or no damage to cells and tissues. Extensive damage can be caused by the rapid ice crystal formation that occurs upon nucleation at lower temperatures (Lee and Costanzo 1998). Reduction of water content in the insect, in preparation for freezing, reduces the possibility of mechanical damage and helps concentrate low-molecular-mass polyol, sugar, and amino acid cryoprotectants. Some of these cryoprotectants may bind water molecules inside cells, thus preventing a critical amount of water loss upon freezing (Storey et al. 1981). Cryoprotectants also assist

stabilization of proteins and cell membranes (Carpenter and Crowe 1988). Accumulation of high levels of cryoprotectant molecules usually occurs as part of cold-hardening for winter (Denlinger and Lee 1998).

Ice formation is generally initiated by the use of one or more of four types of ice nucleators. Inoculative freezing relies upon high moisture content within the overwintering site. This chain reaction of ice formation may gain access to the hemolymph and tissues through natural body openings, such as spiracles, or directly through the integument (Steigerwald et al. 1995). Ice-nucleating bacteria and fungi have groups of ice-nucleating proteins in the cell wall structure that allow them to be efficient at ice nucleation. Several of these ice-nucleators exist in the guts of some insects. This may be the reason that ability to supercool increases in some insects after gut emptying (Lee et al. 1995). Ice-nucleating proteins and lipoproteins are carried in the hemolymph of many freeze-tolerant insects. Production of these proteins usually occurs as part of seasonal cold-hardening (Duman et al. 1985). The most recently discovered group of ice-nucleators is composed of crystalloid inorganic compounds (Lee and Costanzo 1998). Tribasic calcium phosphate spherules, which ice-nucleate in larval *Eurosta solidaginis* Fitch (Diptera: Tephritidae), disappear at the transition to pupa. This disappearance coincides with an increase in supercooling ability. Other such deposits are found in some insects during diapause or overwintering (Mugnano et al. 1996).

2. Supercooling

The supercooling capacity of an insect, usually measured in terms of its supercooling point, is an important parameter in insect winter survival. The supercooling

point refers to the temperature to which a liquid chilled below its melting point can be brought before freezing. An insect's supercooling point is the lowest temperature measured before the release of latent energy, expressed as heat, upon crystallization of hemolymph fluid (Fig. 1.4). Small volumes of water naturally supercool to temperatures far below 0°C. Supercooling ability has an inverse relationship with volume and a direct relationship with exposure time (Zachariassen 1992). Insects may be viewed as small bags of water and thus usually do not freeze at 0°C (Lee 1989).

During summer *Rhabdophaga strobiloides* Osten Sacken (Diptera: Cecidomyiidae) larvae have supercooling points of –26.5°C. During winter they produce glycerol, which allows supercooling points of –56.1°C (Miller and Werner 1987). Glycerol, sorbitol, mannitol, ethylene glycol, ribitol, erythritol, inositol, fructose, trehalose, and glucose are low-molecular-mass molecules that act as anti-freeze compounds in freeze-intolerant insects instead of as cryoprotectants as in freeze-tolerant insects (Duman et al. 1995). Some water loss, as previously mentioned, helps concentrate these antifreeze compounds in the insect hemolymph, thus better protecting it from freezing. Thermal hysteresis proteins are non-colligative acting molecules that increase the gap between melting and freezing points. Production of thermal hysteresis proteins, upon exposure to short photoperiod and low temperatures, can lower supercooling points in some insects 5 to 6°C below their hemolymph melting point (Duman 1977). The lowered supercooling point seems to stem from an ability of these proteins to override ice nucleators in hemolymph. These hysteresis proteins also may occur in association with epidermal tissue and thus prevent inoculative ice formation in the insect (Tursman and Duman 1995).

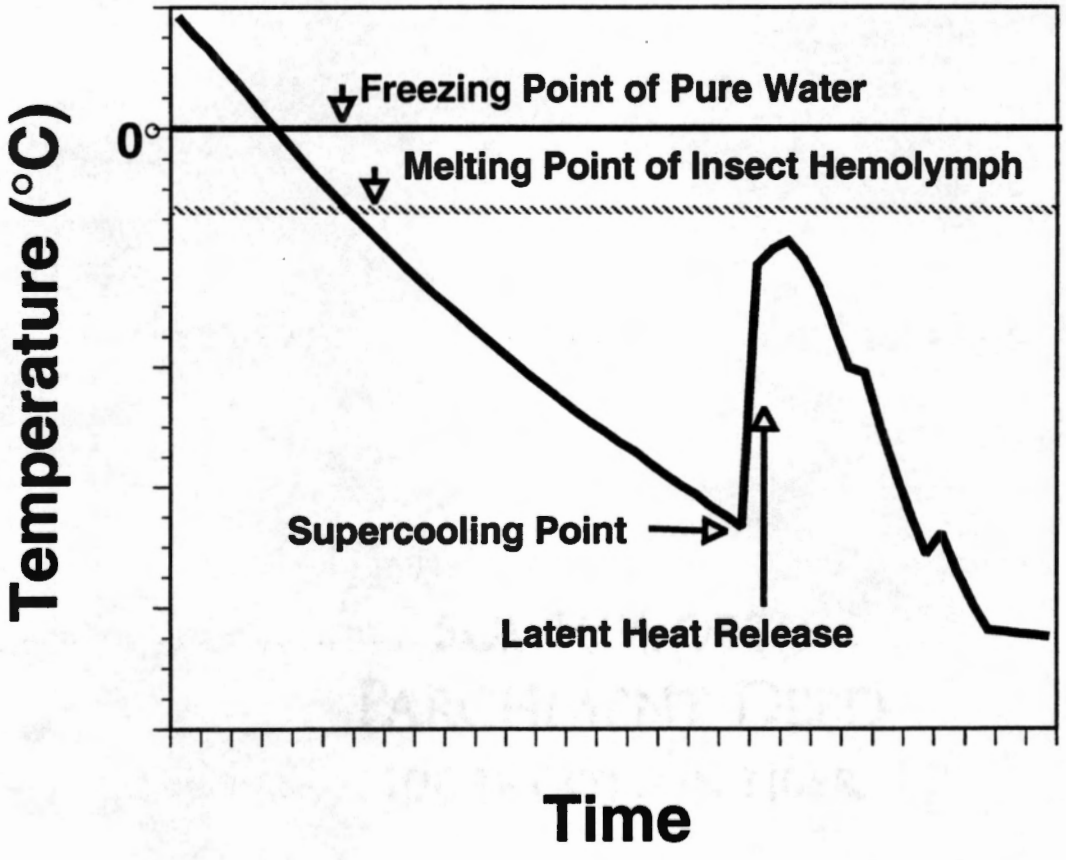


Figure 1.4. A general diagram of the stages insect fluids pass through when the insect is exposed to temperatures that are low enough to cause the insect to freeze (at the supercooling point). Modified from Lee 1991.

3. Acclimation

Traditionally, acclimation develops from the slow, long-term exposure to progressively lower or consistent low temperatures, as in a gradual change of seasons (Baust and Nishino 1991). This process may coincide with particular life stages of an insect such as diapausing pupae. Diapause, a dormant state of lowered metabolism, when associated with acclimation, tends to give increased cold-hardiness ability at the time when diapause is expressed (Denlinger 1991). The polyol, sugar, and amino acid cryoprotectants previously mentioned often result from acclimation, but their method of protection is not always known (Morgan and Chippendale 1983). Vitrification may occur in insects with high concentrations of sugars. It may prevent cell and tissue damages seen with ice formation, because the solidified liquid remains amorphous (Wasylyk et al.1988).

Rapid cold-hardening is a quick response to a sudden drop to a low temperature that is above the insect's freezing point (Yocum et al. 1994). After an initial exposure to low temperatures, production of stress proteins, which reduce or prevent later damage when an insect is exposed again to low temperatures, may occur (Denlinger et al. 1991). The possible actions of these cold-stress proteins are the elimination or rebuilding of damaged enzymes, protein folding, and or oligomeric complex assembly, but the exact mechanisms are not known (Denlinger and Lee 1998).

iii. Imported Fire Ant Cold Resistance

Ice-nucleation and supercooling are generally of concern for organisms that overwinter at sub-zero temperatures (Denlinger and Lee 1998). Considering their mostly

tropical native range, imported fire ants would not be expected to have developed cold weather survival mechanisms, such as winter dormancy or freeze resistance. Indeed, overwintering fire ants are non-dormant, non-freeze tolerant adults (Francke and Cokendolpher 1986). Even though imported fire ants may be active within their mound and forage on warm days during winter, they cannot be considered true winter-active insects, since they cease activities outside of the mound at 10 to 15°C (Cokendolpher and Phillips 1990, Porter and Tschinkel 1987). It had been hypothesized, due to their non-freeze tolerant status and reported supercooling points of approximately –6°C, that the imported fire ants would not infest areas north of a –12°C minimum January isotherm due to biological restrictions in the colder environment (Diffie et al. 1997). Thus, expansion of the range in the United States into northern territories beyond this isotherm has led to much speculation on the final limits of imported fire ant range due to winter mortality and possible mechanisms of winter survival.

a. Behavioral Response

Based on behavior, imported fire ants have a well-developed sensitivity to temperature and humidity gradients (Cokendolpher and Phillips 1990, Porter and Tschinkel 1987), particularly when tending brood (Porter and Tschinkel 1993, Cokendolpher and Francke 1985). During their normal active season, imported fire ants move with the daily temperature changes within the column of the nest soil to take advantage of optimal temperatures (Pinson 1980). It has been suspected that when exposed to long-term cool temperatures, the ants move deeper in the mounds to avoid cold. The ants come back to upper layers of the soil column and return to normal activity,

such as foraging, whenever temperatures are suitably warm. Further evidence of their movement deeper into the nest has been reported by Morrill et al. (1978), as the ants were not found in nest soil above ground during winters in Georgia. The cessation of outside activity at the moderately high winter temperatures (10 to 15°C) would work to prevent outside exposure of the ants to incapacitatingly low temperatures (Cokendolpher and Phillips 1990). Reports of the presence of dead ants in the upper portions of mounds have indeed coincided with rapid temperature drops that could have placed the ants in cold coma before possible movement deeper into the nest (Diffie et al. 1997, Morrill et al. 1978, Green 1959).

Increased queen survival has been found in larger colonies leading to speculation that the ants may cluster around the queen to provide a thermal buffer (Kaspari and Vargo 1995). This theory is supported by findings of Callcott et al. (2000) and Green (1959) who reported that the larger, more mature colonies were more prevalent after harsher winter conditions.

Fire ants shelter in their nests; therefore, nest construction and location are factors in avoidance of lethal low temperatures. Hubbard and Cunningham (1977) found that imported fire ant mounds had a long axis that maximized solar exposure to the broader east and west sides of the mounds. Colonies in the costal plain of Georgia more frequently survived winter in locations exposed to substantial sunlight, while colonies in shaded locations became inactive (Morrill et al. 1978). Nests in suitable microclimates or in association with protective structures may provide further escape from extreme temperatures. Ants collected in January 2000 from Sequatchie Co., TN, were active within

their mounds, which were built in and around moist tree stumps (pers. observ.). Baust (1976) found that wet tree stumps exhibited slow fall cooling and rapid thaw, and Danks (1978) recorded temperatures near 31°C on the sunny side of a tree stump and 2°C on the shady side during January in Ottawa, Canada. Manmade structures, such as buildings and housing for electrical equipment, offer warmth and protection from unfavorable weather. After record low winter temperatures in Georgia, only those colonies with mounds located in protected areas survived (Morrill et al. 1978).

b. Physiological Response

Behavioral responses to low temperatures and the qualities inherent in the construction of the imported fire ant nest may explain much of their ability to survive in relatively cold regions. Nevertheless, several studies also have examined acclimation abilities and supercooling point of imported fire ant workers to determine if physiological barriers to cold injury also exist.

1. Acclimation Testing

Acclimation tests have been conducted both in laboratory and field settings and have yielded indefinite results. Winter collected *S. invicta* maintained for two weeks at 12, 22, or 32°C showed a significant effect of maintenance temperature in their temperature preferences. Summer-collected individuals did not show significant effects from prior maintenance temperatures on preferences (Cokendolpher and Francke 1985). Supercooling tests conducted with spring- and summer-collected *S. invicta* and *S. richteri* after maintenance at 12, 22, or 32°C demonstrated no effects on supercooling ability due to acclimation (Francke et al. 1986). Field tests on *S. invicta* supercooling point

conducted every two weeks from mid October to mid February near Lubbock, Texas revealed a significant effect of test date, but the maximum difference between means was less than 2°C (–4.5 to –5.9°C) demonstrating no definite acclimation effect (Taber et al. 1987). Spring- and summer-collected colonies of *S. invicta,* which were maintained in the laboratory at 22°C for approximately one month and then at 10, 5, and 0°C for one week, showed no significant difference in supercooling points of ants from the different treatments (Landry and Phillips 1996).

2. Supercooling Point Testing

Supercooling point determinations for imported fire ants have either attempted to define *S. invicta* supercooling ability in response to acclimation or compare them with the supercooling abilities of other *Solenopsis* spp. Tests to evaluate the effects of acclimation treatment on supercooling ability demonstrated that *S. invicta* has a supercooling point that is fairly stable within a 2°C or less range regardless of prior temperature exposure (Taber et al. 1987, Landry and Phillips 1996). Yet, the laboratory-maintained colony for the experiment by Taber et al. (1987) displayed a range of supercooling points from approximately –14.5 to –9°C, while field colonies tested within a range of –6 to –4.5°C. The supercooling points for the experiment by Landry and Phillips (1996) ranged from –6.9 to –6.0°C.

Various life stages of the different castes of the fire ants *S. aurea, S. geminata, S. invicta, S. xyloni,* and *S. richteri* were tested for supercooling point (Francke et al. 1986). Among the groups tested, the pupal stages had the lowest supercooling points, as would be expected due to the emptying of gut contents and subsequent loss of efficient ice-

nucleators. The worker larvae and pupae supercooled to lower temperatures than either female or male larvae and pupae, which would be expected due to the inverse relationship of volume and supercooling ability as previously mentioned. *S. invicta* small, medium, and large worker adults supercooled to –9.4, –8.0, and –6.8°C, respectively. Only small, medium, and large adult workers were tested for *S. richteri* (–6.3, –6.2, and –6.2°C, respectively). Supercooling experiments conducted by Diffie and Sheppard (1989) examined the supercooling points of adult alate and large worker castes of *S. invicta, S. richteri,* and *S. invicta* x *richteri* hybrids. *S. invicta* male alates supercooled to warmer temperatures than the hybrid or *S. richteri.* There was no significant difference among female alates in the three groups. The *Solenopsis* hybrid workers supercooled to warmer temperatures (–5.9°C) than either the *S. invicta* or *S. richteri* (–6.6 and –7.0°C, respectively).

Neither the acclimation nor supercooling ability studies demonstrated improved cold tolerance of *S. richteri* over the more tropical *S. invicta.* With indications of possible acclimation ability and lower supercooling points for *S. invicta* workers, past studies suggest *S. invicta* have a better ability to survive low temperatures than the more temperate *S. richteri.*

C. Objectives

Several factors served as justification for the studies reported here. The imported fire ants continue to expand northward as evidenced by their increasing quarantine zone in

TN (see Fig. 1.2). A decade has passed since the last study was conducted on the supercooling abilities of the imported fire ants. Previous testing has focused on the ability of the fire ants to supercool to avoid freeze injury, with no clear indication that this parameter is adequate to measure imported fire ant low-temperature survival. Previous studies have not addressed possible differences among the imported fire ant species and their hybrid in ability to withstand low temperatures above supercooling point. Lastly no prior studies have examined the effect of the biological control agent *T. solenopsae* on the ability of imported fire ants to survive low temperature.

The objectives of this research were as follows:

1. To determine if supercooling ability of *S. richteri, S. richteri* x *invicta* hybrid, and *S. invicta* has increased through a decade of northward expansion,
2. To establish baseline supercooling points for Tennessee populations of *S. richteri* and *S. richteri* x *invicta* hybrid,
3. To examine the effects of season on cold-tolerance of Tennessee populations of *S. richteri* and *S. richteri* x *invicta* hybrid,
4. To explore possible effects of *T. solenopsae* infection on low temperature survival,
5. To discern species differences among imported fire ant species' ability to survive prolonged low temperature exposures, and
6. To study the possible use of ice-nucleating bacteria as control agents for imported fire ants in their northern range.

CHAPTER II

Materials and Methods

A. Collection and Maintenance

i. Colony Collection

Colony collection occurred through shoveling an active fire ant mound into an 18.9-ℓ bucket. Early in the morning when the sun is first heats the mound, the ants move the brood and queen to the upper portions of the mound for optimal use of insolation. Collection performed at that time allows collection of complete colonies with brood and queen. A band of Fluon® (Asahi Glass Fluoropolymers USA, Inc., Chadds Ford, PA) coated the inner surface of all fire ant containers to prevent ant escape, and screen lids ensured alates would not escape by flying. Usually 8, but no fewer than 5, colonies were collected at a time for each species/infection group. After containers holding active ant nests were returned to the laboratory, they were placed in moats of soapy water as a further deterrent to ant escape. An intravenous drip-tube setup, similar to the one described by Banks et al. (1981) and dripping at a rate of approximately one drop every 2 s, was used to separate ants from nest soil. Under a slow constant drip, fire ants form a floating mass of ants with the brood and queen protected in the middle. Floating ants were scooped out with a slotted spoon and placed in plastic housing containers (40.6 x 28.5 x 17.5 cm or 34.5 x 24 x 12.5 cm) (Rubbermaid®, Wooster, OH) coated with Fluon® as previously mentioned. After ant separation, nest soil was autoclaved and disposed in compliance with an APHIS-PPQ permit for transport of fire ants. Colonies within each

species/*T. solenopsae*-infection group were assigned identification numbers to allow comparison of results for the same colony throughout the studies.

ii. Colony Sources

S. richteri, the black imported fire ant, and the hybrid colonies were collected from counties within Tennessee's southern border, because these counties are part of the northern most portion of the imported fire ant range in the northern hemisphere. These sites also were convenient as they are locations where *T. solenopsae* and/or phorid flies (*Pseudacteon* spp.) have been introduced as biological control agents through the Tennessee Fire Ant Project. *S. richteri* were collected from the Ames Experiment Station in Fayette and Hardeman Counties, Tennessee, on Nov. 1, 1999 (fall *S. richteri*), and May 25, 2000 (spring *S. richteri*). Hybrid colonies collected on Nov. 29, 1999 (fall hybrid), and Jun. 9, 2000 (spring hybrid) came from Bradley Co., Tennessee. Additional hybrid colonies from Sequatchie Co., Tennessee, were collected on Jan. 11, 2000. All Tennessee colonies were mature and obtained from areas that had been infested for more than one year, thus, it is assumed that the colonies were either exposed to, or originated from mounds exposed to, local winter conditions.

Spring-collected *S. invicta* colonies infected with *T. solenopsae* were obtained from Alachua Co., Florida, on Mar. 21, 2000 (infected spring *S. invicta*). Uninfected *S. invicta* colonies were collected from Taylor Co., Florida, on Mar. 20, 2000 (uninfected spring *S. invicta*). Five colonies of uninfected and five colonies of *T. solenopsae*-infected *S. invicta* were collected Dec. 13 through 16, 1999 (winter infected and uninfected *S.*

invicta). These colonies were also tested for supercooling point, but they were only partial colonies and had been in laboratory conditions a month prior to testing.

iii. Colony Housing and Maintenance

Artificial nests were provided in the plastic containers to give ants a protected humid nesting environment. This was accomplished either by using 20 x 150-mm test tubes with moist cotton at the end or 150 x 15-mm petri dishes filled about 5 mm deep with Castone® (Dentsply-Trubyte, Jacksonville, Florida). Sometimes, the ants would make their own nests by boring through the cotton plug in water tubes. Test tube nests were replaced as they dried, whereas petri dish nests were re-wetted as needed. Test tubes (20 x 150 mm) of distilled water plugged with cotton were placed in the containers as a water source. Ants were fed to repletion on a 15% honey - 15% dog food agar diet and insects (*Tenebrio molitor* L. [Coleoptera: Tenebrionidae], *Popillia japonica* Newman [Coleoptera: Scarabaeidae], *Galleria mellonella* L. [Lepidoptera: Pyralidae], and *Periplaneta americana* L. [Blattaria: Blattidae]).

iv. Species Verification

Gas chromatographic (GC) analysis of venom and cuticular hydrocarbons was used to identify species. *Solenopsis richteri* and *S. invicta* show distinctly different patterns in both venom and cuticular hydrocarbons when tested. Hybrid ants have blends of both parent patterns. Sometimes either the venom or the hydrocarbon will match a parent pattern but not both criteria (R.K. Vander Meer, pers. comm.), thus both criteria were used. The ants in colonies collected from Florida, an area where hybrid imported

fire ants are not found, were not included in the GC testing, as they are known to be *S. invicta*. All Tennessee colonies used in the experiments were tested for species verification.

Groups of approximately 20 ants from each colony were placed in vials containing 1 ml hexane from 2 to 24 h to remove sufficient venom and hydrocarbon components. Ants were then removed from the vials, and the hexane evaporated prior to mailing. All samples were analyzed at the USDA – ARS, CMAVE laboratories in Florida, where equipment and expertise were available. Once received in the USDA laboratory, hexane was added to the vials and the reconstituted venom/hydrocarbon hexane solution was transferred to GC sample vials. Samples were then analyzed by gas chromatograph (Varian 3700, Varian Associates, Walnut Creek, CA) equipped with a split-splitless injector, a capillary column (Agilent Technologies; J & W Scientific Incorporated, Folsom, CA; DB-1, 30 m, 0.32 mm i.d., 0.25 μm film thickness), and flame ionization detector. The injector and detector were set at 300°C; the oven temperature was programmed to rise from 120 to 285°C at the rate of 5°C/min, and then held at 285°C for 5 min. Hydrogen was used as the carrier gas and nitrogen was used as the makeup gas. The data were analyzed using PE Nelson Turbochrom Navigator 6.1.0.1FO4 (Perkin Elmer Corp., Norwalk, CT).

B. Supercooling Tests

i. Test Subjects

Five colonies of each collection group were selected for each of the tests. Colonies numbered 1 through 5 were tested for the following: fall *S. richteri*, spring *S. richteri*, fall hybrid, spring hybrid, infected winter *S. invicta*, uninfected winter *S. invicta*, and infected spring *S. invicta*. The spring uninfected *S. invicta* colonies used were those numbered 1, 3, 4, 5, and 8. Ten small and ten large workers were collected from each colony and individually frozen to collect supercooling point data. Size determination was relative to other individuals in a colony, as colonies differed in their composition of individual size ranges. Small and large ants were selected at random from the smallest and largest ants visible in the colony, respectively. Large ants had head capsules >0.9 mm in diameter and small ants <0.9 mm. Based on preliminary data, variation in supercooling increased after one or more months of exposure to laboratory conditions, thus ants were tested as soon after collection as possible, within 1 wk for Tennessee colonies and 2 wk for Florida colonies. Ants collected in fall 1999 were tested at 1 wk after collection, maintained in the lab for 3 mo, and then retested to determine the effects on supercooling point from long-term maintenance under laboratory conditions.

ii. Supercooling Test Procedure

Ants were attached by means of petroleum jelly to copper-constantan thermocouples (Omega Engineering, Inc., Stamford, CT) (Landry and Phillips 1996,

Diffie and Sheppard 1989, Francke et al. 1986). Thermocouples were either 0.1 mm or 0.01 mm diameter. Early tests revealed that major workers and alates broke the 0.01 mm thermocouple wire and the sensitivity to temperature change of minor workers was lost when the larger 0.1 mm diameter wire was used. Thus, large ants were tested on the large thermocouples and small ants were tested on the small thermocouples. Due to the use of different sized thermocouples, supercooling temperatures of small workers were not directly compared with large workers except for in the early tests mentioned two sentences before.

Each ant/thermocouple array was inserted into a 48 x 15 mm glass vial to protect ant attachment and buffer temperature change from more rapid change in the freezer. Thermocouples were attached to a 3 mm-diameter dowel protruding through the center of a small cork. This procedure was done to hold the ant/thermocouple array in place in the center of the vial. Up to four ants were tested simultaneously by placing arrays in a test tube rack in a Galaxy chest freezer (Sears, Knoxville, Tennessee). Freezer temperature was set at –24°C giving the thermocouples in the vials a temperature drop rate of approximately 5.6°C/min during the first 4 min and then slowing to approximately 3.5°C/min by 10 min. Ant temperature changes were recorded through a Campbell Scientific CR10 data logger (Logan, UT), after the thermocouple located on the primary port read 1°C. An IBM 486 personal computer, equipped with Campbell Scientific PC208W data logger support software, allowed temperature changes to be monitored as they occurred and was used to store and retrieve the data logger files.

The supercooling point of each insect was recorded as the lowest reading reached before the release of latent energy. Data from each active thermocouple were designated

by a different colored line on the computer screen and graphed as the temperature decreased (Fig. 2.1). The data logger itself also housed a temperature gauge, which was used as a room temperature reference point and included in the data graphed on the screen. The energy release, upon surpassing the supercooling point, was graphed as an abrupt jump to a temperature several degrees warmer than the supercooling point. After all ants currently in the freezer surpassed their supercooling points, the ants were removed and saved for head capsule measurement and infection verification.

iii. Head Width Measurement

Head capsules of ants have been closely correlated with their body size (Calabi and Porter 1989). Thus, head capsules were measured to determine if a correlation existed between ant size and supercooling point. Head capsules were measured with a wedge micrometer (Porter 1983), wherein the head is moved down a graduated groove using forceps until it cannot be pushed further. To form this groove two microscope slides were mounted with epoxy to form a narrow "V" on a sheet of hard plastic. A Mitutoyo® dial caliper (Tokyo, Japan) was then used to measure 0.1 mm intervals of groove space, and marks were made accordingly. A wedge micrometer with cover slips instead of slides was constructed to measure heads less than 1.0 mm. Slide thickness in the larger wedge micrometer prevented further manipulation of <1.0-mm diameter heads.

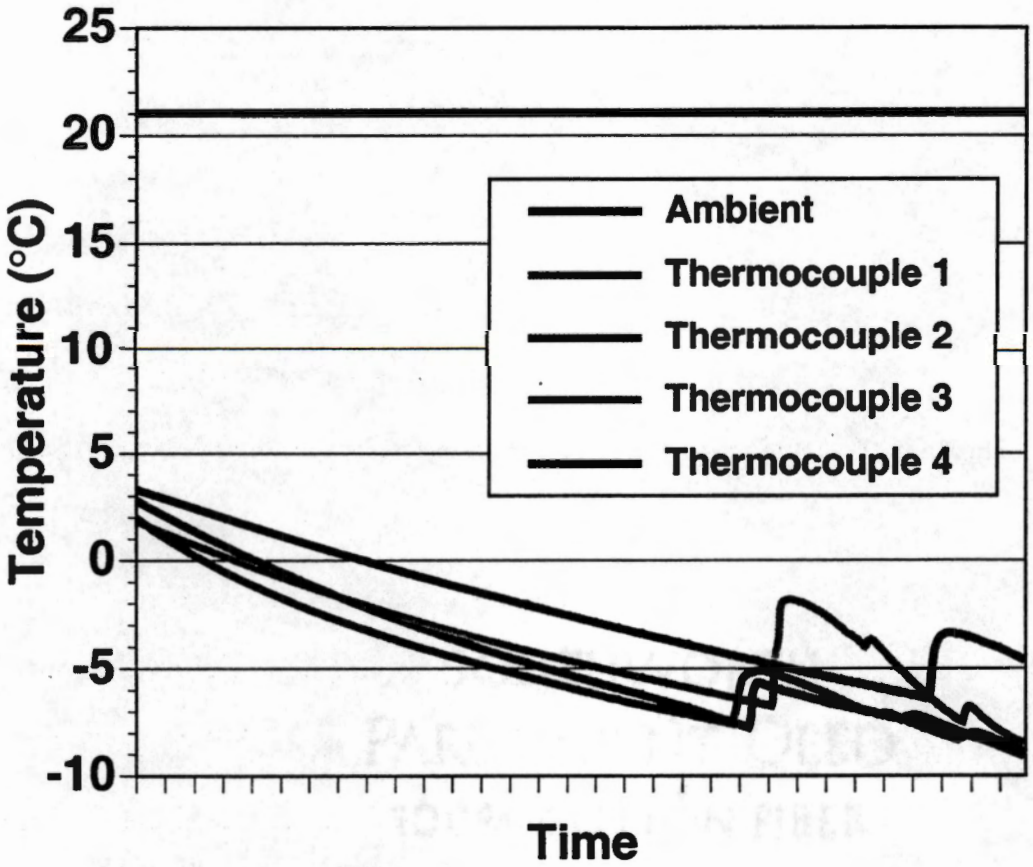


Figure 2.1. Graphic representation of the data points recorded by the CR10 datalogger as displayed on the computer screen during supercooling point testing.

iv. Determination of *Thelohania solenopsae* Infection

After use in supercooling determination, *S. invicta* were checked for *T. solenopsae* infection. A wet mount slide of the ant's abdominal contents was prepared and examined under a microscope at 400x magnification. Several fields of vision were observed per slide. The slides were inspected immediately after preparation for presence of protozoan spores, allowed to dry, and then re-wetted, if no spores had been immediately visible. Drying of the slides promoted aggregation of materials floating in the fluid and made it easier to spot spores. Ants were considered infected when at least two meiospores were observed, otherwise the ants were recorded as uninfected.

Whole live ants as used in supercooling tests do not show external signs of *T. solenopsae* infection. Therefore, infection status could not be confirmed prior to use in supercooling tests. Furthermore, because some ants from infected colonies could not be confirmed as being infected with *T. solenopsae* (see Appendix A for colony infection rates), it was important to determine if individual infection had an effect on supercooling. Thus, results of confirmed infected ants versus apparently uninfected ants originating from infected colonies were compared.

v. Statistical Analysis

Supercooling point data were analyzed with the SuperANOVA (Abacus Concepts, Inc., Berkeley, CA 1989) program. One-way ANOVA on a type III sums of squares followed with mean separation by Student-Newman-Keuls procedure at a $P = 0.05$ level of significance was performed for all groups compared. Supercooling points of small and large ants as measured using small and large thermocouples were analyzed to

determine if significant differences existed between measurements with the two thermocouple sizes. Groups of ants from within the same size category are compared in all other analysis. Spring-2000-collected ants were compared with analogous fall-1999-collected groups to examine seasonal differences. Species and infection level effects were examined for those collected in the same season.

Further analysis of supercooling point data was done on each ant group after determination of head size. Comparisons were made across species within the same size and collection group to determine if a significant difference existed among groups within a limited head capsule size range. Comparison across species groups was performed both with all ants used in the supercooling test, and using only data from those with head capsules in either the 0.70 - 0.75 mm range (small ants), or 1.25 - 1.40 mm range (large ants). Head capsule size also was used in correlation analysis with supercooling points. This was done to determine if ant size, as represented by head capsule width, influenced supercooling points.

C. Extended Low Temperature Exposure

i. Test subjects

When possible, the same colonies used in supercooling tests were used in the extended cold exposure tests. Colony selection was based on the five most populous spring-collected colonies of *S. richteri,* hybrid, infected *S. invicta*, and uninfected *S. invicta*. This experiment was repeated, but some of the colonies had to be substituted in the second test due to low population. Ants in the first run of these experiments came

from the following colonies: uninfected *S. invicta* #1, 3, 4, 5, 7; infected *S. invicta* #1,2,4,5,7; and *S. richteri* and hybrid #1-5. Between the first and second runs of this experiment the following colonies were replaced: uninfected *S. invicta* colonies #7 and #3 were replaced with #2 and #6. Infected *S. invicta* colony #4 was replaced with #3. *S. richteri* colony #1 and hybrid colony #5 were replaced with their respective #6 colonies. All ant groups used in this experiment were collected in the spring.

ii. Extended Cold Assay Procedures

Extended cold exposure experiments were conducted on groups of usually 20 to 30 (but ranging from 14 to 85) worker ants per test tube. Test tubes (20 x 150 mm) were prepared with a Fluon® ring around the upper interior wall of the tube to prevent ant escape, and a base of moist Castone® to maintain tube humidity at uniform levels. After ants were manually loaded into the tubes, caps that allowed gas exchange were placed on the test tubes to prevent debris from entering. The ant-filled tubes were placed in test tube racks prior to placement in a refrigerator.

For each colony in the experiments, one test tube was prepared for each of eight sampling days, for each of the three temperature regimes, totaling 120 test tubes per test group. The ants of each test tube were discarded after use in mortality determination. Re-sampling was avoided so observations would be based on a constant exposure to the temperature regimes. Ants were sampled on days 1, 2, 3, 5, 7, 10, 13, and 15. Due to space limitations, ants were placed in the refrigerators in groups of 4 sample days, with tubes for days 7-15 being placed in refrigerators after tubes for days 1-5 had been removed. The racks of ant tubes were placed inside styrofoam packing coolers with 4cm-

thick walls. Sand, chilled in the appropriate refrigerator, was added over the tops of the test tubes to the base of the coolers at a depth of approximately 2 cm to provide further temperature buffer against the refrigerator cycle.

Three temperature regimes were used (mean ± std, +4 ± 0.5°C = positive, 0.5 ± 0.5°C = zero, and –4 ± 0.3°C = negative). All temperature regimes were above the fire ant supercooling points as determined in previous experiments. Exposure to these temperature regimes lowered the metabolic rate and usually induced cold coma within 24h. While in cold coma, ants could not move and thus no food or water was provided. A room temperature control, set up as described previously, was used in preliminary tests, but later eliminated from the experiment. Ants at room temperature had normal metabolic rates and died within the fifth test day due to lack of food or water. StowAway® (Onset, Pocasset, MA) data logger units were used to record temperatures in each cooler. Coolers were left open for an initial period of 4.5 h, after which lids were placed on the coolers. This open period was necessary to facilitate the temperature drop in the test tubes and coolers. Closing the coolers after this period maintained uniformity at the desired temperature.

On sample days, ant tubes were removed from the coolers for counting of live, moribund, and dead individuals. Ants were considered live if they could return themselves to an upright position and walk after being placed on their backs. The moribund designation was given to individuals that showed any movement, but could not turn themselves over after being placed on their backs. Dead ants showed no movement. In prior trials, ants designated as moribund did not recover 24 h after removal from the refrigerators. These ants were subsequently included as dead in data analysis.

Ants at the 0.5 and –4°C regimes were transferred from the test tubes into lidded plastic cups at room temperature immediately after removal from their respective temperatures. In previous testing, some ants in these groups, while under cold coma, drowned in condensation that formed in the test tubes. Transfer to the room-temperature plastic cups avoided this drowning and revived ants quicker than when they were left in the cold test tubes. The ants kept at the +4°C temperature regime, if experiencing cold coma, recovered within minutes of placement at room temperature and were not subject to the condensation problem; thus they were counted first. Recovery periods of 2 and 3 h were given to allow ants at 0.5 and –4°C temperatures, respectively, to recover from cold coma. After the mortality counts, the –4°C ants for the *T. solenopsae*-infected colonies also were used to determine percent colony infection of those not previously sampled through supercooling. These ants were returned to their plastic cups and stored in the freezer until slides could be prepared, as previously discussed, for 10 ants from each of these colonies. All other ants, once counted, were frozen and discarded.

iii. Statistical Analysis

Percent mortality data were arcsine-transformed (arcsine [sqrt (% mortality/100)]), and ANOVA analysis was performed, as previously described for supercooling data. No ant size categories were used in the analysis of the extended exposure test, as worker ants were placed in the test tubes regardless of the individual size. All four test groups were compared within the same temperature regime. Probit analysis using POLO - PC© (LeOra Software 1987) was used to determine LD_{50} of cold

exposure as measured in the number of days for each ant group within each temperature regime.

D. Ice-Nucleating Bacteria Tests

Due to difficulties encountered with these experiments, only preliminary tests were conducted on ants exposed to ice-nucleating bacteria. Initially, the ice-nucleating properties of bacterial isolates were tested. Since ice-nucleators promote crystal formation (freezing) at warmer temperatures, ice-nucleating ability was determined by an increase in supercooling point of the test subject. Bacterial isolates displaying ice-nucleation capacity were applied to ants and the supercooling points of these ants were tested. Supercooling point determinations for both bacterial suspensions and treated ants were carried out as previously described.

i. Test Subjects

First attempts to find an ice-nucleating bacterial isolate included an isolate of *Pseudomonas syringae* pv. *syringae* (Pss), the bacterial species used on *S. invicta* by Landry and Phillips (1996); several isolates of *Pseudomonas fluorescens* (Pf) Migula; and an isolate of *Bacillus thuringiensis* pv. *israelensis* (Bti) de Barjac (Bacillales: Bacillaceae) as a non-ice-nucleating control. A second group of trials included isolates of *Bacillus sphearicus* (Bs) Meyer and Neide, *Pseudomonas syringae* pv. *phaseolicola* (Psp) (Burkholder), *Pseudomonas syringae* pv. *tabaci* (Pst) (Wolf and Foster), and Pss. Hybrid

fire ants from a colony collected in Sequatchie Co., Tennessee, were used in the bacterial application tests.

ii. Bacterial Growth and Preparation

All tested bacteria were grown in 250-ml Erlenmeyer flasks containing 150 ml of nutrient-yeast extract broth. After incubation for four to five days, bacterial growth sufficient to turn the broth opaque was attained. The bacterial broth suspensions were then spun down at 3500 rcf for 30 min in a Heraeus Instruments Labofuge 400® (Newtown, CT) centrifuge until a tightly packed bacterial pellet was formed. The supernatant was discarded and the bacterial pellet was re-suspended in 50 ml sterile deionized water, which resulted in an opaque bacterial suspension.

iii. Bacterial Suspension Testing

Bacterial isolates were first tested for ice-nucleating properties. A 0.05 ml aliquot of the bacterial suspension was pipetted into a sterile 12 x 75 mm polystyrene culture tube. A 0.1 mm thermocouple held in place by a cork was inserted in the liquid, and the tube/thermocouple assembly was placed in a test tube rack in the freezer. One culture tube of pure water and three tubes of bacterial suspensions were tested simultaneously. The supercooling points of the liquids were recorded via the same apparatus described previously for the supercooling point tests. Thermocouples were sprayed with a 70% ethanol solution and wiped between isolates. Ten samples were tested for each bacterial isolate.

iv. Bacterial Application to Ants

Based on results from the bacterial testing, Psp and Pst showed promise as ice-nucleators and were used in the ant application tests. Three techniques were used for applying bacterial solutions to the ants: mist spraying, submerging, and feeding.

a. Spray Application

The spray test attempted to follow the methods in Landry and Phillips (1996). Three treatments were used: Psp, Bti, and sterile deionized water. Tween 80® (Difco, Detroit, MI) and vegetable oil were added at 5 ml each to 100 ml bacterial or sterile water. Tween 80® and vegetable oil were added to dissolve ant cuticular wax and aid in bacterial adhesion, respectively (Landry and Phillips 1996). Suspensions were agitated using a Thermolyne Maxi Mix II® mixer (Dubuque, IA) until homogenized.

Ants removed from a colony were split into three groups of approximately 50 individuals and placed in Fluon®-lined plastic containers (22 x 14 x 7 cm). The ants were then sprayed with 1 ml of the Psp, Bti, or water, with tween and oil. After drying, ant supercooling points were determined as previously described. Ten to twelve ants were tested for each treatment.

b. Submergence Application

The submergence tests used Pst, Bs, and sterile deionized water. Tween 80® and vegetable oil were mixed with each suspension in the same proportions as in the spray test. Sterile polystyrene culture tubes (12 x 75 mm) were modified into submergence chambers by removing the closed end and melting a 1-cm^2 piece of wire mesh over the new opening. This procedure was repeated for the caps. The submergence chambers were then placed in the ant colony container for a few minutes while ants entered. A 25 ml-

graduated cylinder was used to hold 15 ml of each suspension. The ant-filled chambers were dropped into the suspensions, allowed to sink until all ants were submerged, and immediately removed to reduce drowning. The cap was removed and the treated ants and chamber were placed on a paper towel inside a Fluon®-lined ant-housing container. After ants were dry, supercooling points were determined as previously described. A second run of this test did not allow for drying time and ants were tested immediately after treatment. Ten to twelve ants were tested for each solution.

c. Feeding Test

A group of approximately 50 workers was removed from the same hybrid colony used for the other bacterial tests and isolated with a moistened nest cell in a Fluon®-lined ant-housing container. These ants were offered 15% by weight honey in a Pst bacterial water suspension as their only water or food source. After a five-day exposure to the solution, ant supercooling points were tested.

CHAPTER III

Results

A. Supercooling

i. Thermocouple Size

Large ants consistently broke the small (0.01 mm) thermocouples; therefore, the use of larger, sturdier (0.1 mm) thermocouples was necessary for the large ants. Tests revealed a significant difference existed due to thermocouple size. Small ants on small thermocouples had a mean supercooling point of -5.9 ± 0.07°C, whereas large ants on small thermocouples had a mean of -5.8 ± 0.07°C. Thus, no significant ($P = 0.7584$, $df = 1$, $F = 0.095$) difference was found between ants of either size when tested on the small thermocouples. Large thermocouples, however, not only showed significant ($P = 0.0430$, $df = 1$, $F = 4.175$) difference between the two sizes of ants (small ants on large thermocouples = -7.4 ± 0.13°C; large ants on large thermocouples = -7.7 ± 0.10°C), but also read the supercooling points 1.5°C or more lower than the sensitive small thermocouple (small ants on both thermocouples [$P = 0.0001$; $df = 1, 45$; $F = 146.883$] and large ants on both thermocouples [$P = 0.0001$; $df = 1, 45$; $F = 209.411$]). It was important to get as accurate a reading as possible, so small ants continued to be tested on small thermocouples, but large ants were tested with large thermocouples. Due to this difference all following supercooling comparisons are only within the same ant size groups in order to keep comparisons to only those ants tested on the same size thermocouple.

ii. Tennessee Groups of Imported Fire Ants

a. Intra-specific Fall-colony Variation

Preliminary supercooling tests run on colonies that had been maintained under laboratory conditions for several months gave inconsistent results both within and among the colonies (see Appendix B for all colony means). For instance, supercooling point varied within small ants of one *S. richteri* colony from –8.0 to –15.2°C. For small *S. richteri* intra-specific colony means, supercooling points ranged from –6.8 to –12.8°C. When new *S. richteri* and hybrid colonies collected in the fall of 1999 were tested one week after collection, the supercooling point showed little intra-colony difference. This is demonstrated by the small *S. richteri* workers, which had a maximum 1.4°C difference between the highest and lowest supercooling points in any colony. These fall colonies also showed no significant difference ($P > 0.05$) in intra-specific colony mean supercooling point. After three-months maintenance in the laboratory, these colonies were retested and some colonies had developed both wider differences between the maximum and minimum supercooling points of individual ants (e.g., 3.5°C in small *S. richteri*) and significant (small *S. richteri* [$P = 0.0031$; $df = 4, 45$; $F = 4.658$], large *S. richteri* [$P = 0.0374$; $df = 4, 45$; $F = 2.791$], small hybrid [$P = 0.0001$; $df = 4, 45$; $F = 9.115$], and large hybrid [$P = 0.0001$; $df = 4, 45$; $F = 13.435$]) intra-specific differences among the colony supercooling point means, as demonstrated by a range from -7.9 ± 0.35°C to -6.5 ± 0.21°C among the small *S. richteri* workers.

b. Fall *S. richteri* and Hybrid

There were significant (small ants [$P = 0.0001$, $df = 1$, $F = 25.610$] and large ants [$P = 0.0018$, $df = 1$, $F = 9.970$]) differences between the supercooling points of fall

S. richteri and hybrid ants (Table 3.1). Mean supercooling points of both small and large ants from *S. richteri* colonies were lower than the corresponding hybrid ants (small *S. richteri* = –6.8 ± 0.07°C, small hybrid = –6.2 ± 0.05°C, large *S. richteri* = –8.6 ± 0.12°C, large hybrid = –8.2 ± 0.10°C). Three months later, when these same colonies were retested, the significant (small ants [P = 0.0001, df = 1, F = 14.773] and large ants [P = 0.0012, df = 1, F = 10.704]) difference between *S. richteri* and hybrid ants persisted. Small and large *S. richteri* supercooled to –7.1 ± 0.13°C and –8.0 ± 0.17°C, respectively, and small and large hybrid ants supercooled to –6.5 ± 0.08°C and –7.5 ± 0.10°C, respectively. Also, when comparisons were made between test dates, the small ants gained supercooling ability and the large ants lost supercooling ability with time spent in the lab. Changes over time were significant for large *S. richteri* (P = 0.0321, df = 4, F = 4.626), small hybrid (P = 0.0001, df = 4, F = 16.145), and large hybrid (P = 0.0001, df = 4, F = 22.682), but not for small *S. richteri* (P = 0.0526, df = 4, F = 3.779).

c. Intra-specific Spring-colony Variation

Contrary to fall colonies tested within the first week, which displayed no significant differences among them, the small spring *S. richteri* and the large spring hybrid colonies displayed significant (small *S. richteri* [P = 0.0005; df = 4, 45; F = 6.132] and large hybrid [P = 0.0423; df = 4, 45; F = 2.701]) differences among their mean supercooling points. However, intra-colony variation in supercooling points of individual ants was relatively low. Also the range between extreme colony means of the small spring *S. richteri* and the large spring hybrids were only 1.5 and 2.1°C respectively. Thus, the inter- and intra-colony variation in the spring colonies was more similar to that

Table 3.1. Mean supercooling points (mean ± sem, °C) for *Solenopsis* ants collected in different seasons, and tested after different periods in the laboratory.

Species*	Collection time†	Test time	Small‡	Large‡
Solenopsis richteri	Fall	1 wk	–6.8 ± 0.07	–8.6 ± 0.12
	Fall	3 mo	–7.1 ± 0.13	–8.0 ± 0.17
	Spring	1 wk	–6.8 ± 0.13	–6.8 ± 0.08
Hybrid	Fall	1 wk	–6.2 ± 0.05	–8.2 ± 0.10
	Fall	3 mo	–6.5 ± 0.08	–7.5 ± 0.10
	Spring	1 wk	–6.9 ± 0.21	–7.5 ± 0.25
Solenopsis invicta uninfected	Winter	1 mo	–8.8 ± 0.35	–7.5 ± 0.11
	Spring	2 wk	–7.6 ± 0.23	–6.8 ± 0.15
Solenopsis invicta infected	Winter	1 mo	–11.7 ± 0.78	–8.3 ± 0.36
	Spring	2 wks	–6.5 ± 0.13	–6.2 ± 0.05

* *S. invicta* was the only species to express infection with *Thelohania solenopsae*. *S. invicta* that were infected were treated as if they were a separate species from those that were not infected.

† Fall *S. richteri* and hybrids were tested 1 wk after collection and then the same colonies were tested again 3 mo later.

‡ Small ants were tested with a 0.01-mm thermocouple. Large ants were tested with 0.1-mm thermocouple.

of the fall colonies than to the colonies maintained in the lab for several months prior to testing.

d. Spring *S. richteri* and Hybrid

The mean supercooling points of small *S. richteri* and hybrids were not significantly ($P = 0.8072$; $df = 1, 98$; $F = 0.060$) different at -6.8 ± 0.13°C and -6.9 ± 0.21°C, respectively. However, large hybrids supercooled to -7.5 ± 0.25°C, which was significantly ($P = 0.0107$; $df = 1, 98$; $F = 6.771$) lower than the supercooling point of large *S. richteri* (-6.8 ± 0.08°C). Among the small ants of the fall and spring *S. richteri* and hybrids, no significant difference existed among the two *S. richteri* collections and the spring hybrid, but the fall hybrid mean was significantly ($P < 0.05$) higher than the other groups. Large ants of both fall-collected S. *richteri* and fall-collected hybrid supercooled to significantly ($P < 0.05$) lower temperatures than either spring-collected group.

iii. Florida Groups of Imported Fire Ants

a. Winter *S. invicta*

A wide range of supercooling points was obtained among the five partial colonies of uninfected and five partial colonies of *T. solenopsae*-infected *S. invicta* which were tested a month after their December collection. A wide intra-colony supercooling point range also was also displayed. Two of the small ant groups from uninfected colonies and three from infected colonies had intra-colony ranges of more than 11°C. This was similar to results obtained with ants maintained in the laboratory for several months prior to testing, as previously described.

Both the small and large ants of the infected colonies supercooled to lower temperatures (–11.7 ± 0.78°C and –8.3 ± 0.36°C, respectively) than the uninfected ants (–8.8 ± 0.35°C and –7.5 ± 0.11 °C, respectively). Based on further analysis after infection verification of individual ants, there was no significant ($P > 0.05$) difference between supercooling points of infected ants (–9.6 ± 0.58°C) and uninfected ants (–10.5 ± 0.75°C) from infected colonies.

b. Spring *S. invicta*

Spring *S. invicta* colonies, which were whole functional colonies tested within two weeks of collection, had significant (small infected [$P = 0.0011$; $df = 4, 45$; $F = 5.503$], large infected [$P = 0.0006$; $df = 4, 45$; $F = 5.963$], small uninfected [$P = 0.0128$; $df = 4, 45$; $F = 3.580$], and large uninfected [$P = 0.0001$; $df = 4, 45$; $F = 8.351$]) inter-colony differences (see Appendix B). Intra-colony ranges were moderately wide with small ants for two of the uninfected colonies and one of the infected colonies surpassing 4.5°C from lowest to highest supercooling point.

Both small and large uninfected ants supercooled to lower temperatures (small = –7.6 ± 0.23°C, and large = –6.8 ± 0.15°C) than infected ants (small = –6.5 ± 0.13°C, and large = –6.2 ± 0.05°C). The fact that uninfected ants supercooled to lower temperatures than infected ants is an opposite trend to that seen in the winter *S. invicta*. Further analysis of data of confirmed *T. solenopsae* infection in individual ants from infected colonies showed no significant ($P > 0.05$) difference between supercooling means of ants confirmed or unconfirmed as infected. The small uninfected ants from infected colonies supercooled to –6.3 ± 0.13°C, while small ants from the same colonies but with confirmed infection supercooled to –6.6 ± 0.20°C. Large ants from infected colonies, but

without confirmed infection, had a mean supercooling point of –6.0 ± 0.12°C, while the mean of those large individuals with confirmed infection was –6.2 ± 0.05°C.

iv. Spring Colony Comparisons

a. All Tested Individuals

Uninfected small *S. invicta* supercooled to a significantly ($P < 0.05$) lower temperature (–7.6 ± 0.23°C) than the *S. richteri* (–6.8 ± 0.13°C) and hybrids (–6.9 ± 0.21°C). Large *S. invicta* (–6.8 ± 0.15°C) were not significantly ($P > 0.05$) different from *S. richteri* (–6.8 ± 0.08°C), but both groups supercooled to significantly ($P < 0.05$) higher temperatures than the large hybrids (–7.5 ± 0.25°C).

b. Ants with Similar Head Capsule Size

Some groups in the cross-species comparison had significantly different head capsule sizes (Table 3.2). Uninfected small *S. invicta* head capsules (0.60 ± 0.008 mm) were significantly ($P < 0.05$) smaller than statistically similar small *S. richteri* (0.76 ± 0.005 mm) and hybrids (0.77 ± 0.008 mm). Large hybrids (1.36 ± 0.009 mm) had significantly ($P < 0.05$) larger heads than statistically similar large *S. richteri* (1.26 ± 0.010 mm) and uninfected *S. invicta* (1.29 ± 0.014 mm). Small ants within the 0.70 – 0.75 mm head capsule range, which included at least 16 data points from each species compared, echoed the results seen previously when all small individuals, regardless of head capsule size, were tested. Among small ants within the 0.70 – 0.75 mm head capsule range, uninfected *S. invicta* supercooled lower (–7.8 ± 0.38°C) than the statistically similar *S. richteri* (–6.9 ± 0.23°C) and hybrids (–6.7 ± 0.13°C). Among large ants with head capsules within the 1.25–1.40 mm range, *S. richteri* and uninfected *S. invicta*

Table 3.2. Supercooling points (SCP) (mean ± sem °C) and head capsule sizes (HS) (mean ± sem mm) for the complete and head-size selected groups of spring-collected *Solenopsis* worker ants. *

Ant Species	Small Ants‡					Large Ants‡				
	Complete Test Group†		Group with Head Size 0.70 - 0.75 mm			Complete Test Group†		Group with Head Size 1.25 – 1.40 mm		
	SCP	HS	SCP	HS	N	SCP	HS	SCP	HS	N
Solenopsis richteri	–6.8± 0.13 a	0.76± 0.005 b	–6.9± 0.23 a	0.73± 0.000	27	–6.8± 0.08 a	1.26± 0.010 a	–6.6± 0.09 a	1.3±0.007	31
Hybrid	–6.9± 0.21 a	0.77± 0.008 b	–6.7± 0.13 a	0.72± 0.003	19	–7.5± 0.10 b	1.36± 0.009 b	–7.7± 0.35 b	1.34±0.007	34
Solenopsis invicta	–7.6± 0.23 b	0.60± 0.008 a	–7.8± 0.38 b	0.71± 0.004	16	–6.8± 0.15 a	1.29± 0.014 a	–6.8± 0.21 a	1.32±0.009	32

*Head-size selected subgroups consist of data from individuals that fell within a head capsule size range that was shared across all three species.

‡ Small ants were tested with a 0.01- mm thermocouple. Large ants were tested with 0.1-mm thermocouple.

† N = 50, for each of these test groups. Five colonies each of *S. richteri, S. richteri* x *invicta* hybrid, and *S. invicta,* were tested. Ten small and ten large worker ants were tested from each colony.

Means within a column followed by the same letter are not significantly different at the 0.05 level of confidence according to Student-Newman-Keuls means separation procedure.

(–6.6 ± 0.07°C and –6.8 ± 0.21°C, respectively) were statistically similar in their supercooling points, which were higher than that of hybrids (–7.7 ± 0.35°C).

v. Head Capsule Measurements and Correlations

a. Species Head Capsule Range

Colonies of *S. invicta* were generally composed of smaller individuals. Small head capsule widths ranged from > 0.40 to < 0.75 mm, and large head capsules ranged from > 0.90 to < 1.45 mm. Infected and uninfected colonies were not significantly different. Small hybrid ants include head width values from 0.60 to 0.87 mm. Widths of large hybrid ant heads ranged from > 1.15 to < 1.50 mm. Heads of small *S. richteri* ants were between 0.73 to 0.83 mm. Large head capsules of *S. richteri* were ≥ 1.07 and ≤ 1.40 mm.

b. Size and Supercooling Point Correlation

Among all ant groups tested for correlation between head capsule size and supercooling point, only small fall hybrids tested after 3 mo in the laboratory were significantly correlated (R = 0.328). A negative correlation between head size and supercooling point existed for this group. All others had no correlation between head capsule and supercooling point.

B. Extended Low Temperature Exposure

The results for the two trials of the extended low temperature exposure tests were not significantly (P = 0.9602; df = 1, 112; F = 0.003) different; thus, the results were

combined for discussion. Significant ($P = 0.0001$; $df = 2, 112$; $F = 427.493$) differences among the three temperature regimes were found with the mortality rates of ants increasing as temperatures decreased. Probit analysis indicated a poor fit of the data to the model tested in all temperatures and ant groups. Nevertheless, lethal doses (LD_{50} = days of constant exposure at a certain temperature needed to kill 50% of the ants) are presented as an indication of differences among effects of temperature regimes on ant mortality.

i. Positive (+4°C) Temperature Regime

During the first five days at the +4°C regime, no significant ($P > 0.5$) differences were observed as all four ant groups had low mortality (less than 15%) (Fig. 3.1). By the seventh day, however, differences were observed among the ant groups. Infected *S. invicta* and hybrid ants had lower mortalities than *S. richteri* or uninfected *S. invicta,* at least through day thirteen. Mortalities at seven and ten days were significantly ($P < 0.05$) different between ant groups with low mortality, the hybrids and infected *S. invicta,* and ants with high mortalities, *S. richteri* and uninfected *S. invicta.* At day seven, hybrids and infected *S. invicta* had 6.6 and 6.0% mortality, respectively, compared with 30.6 and 35.5% mortality for *S. richteri* and uninfected *S. invicta,* respectively. At day ten the mortalities were 17.7, 28.3, 52.7, and 55.8% for hybrids, infected *S. invicta, S. richteri,* and uninfected *S. invicta,* respectively. By the end of the test at day fifteen, no group had reached 75% mortality.

The probit-analysis estimated LD_{50} was beyond the final test date, day fifteen, for both the hybrid and infected *S. invicta;* therefore, these LD_{50} values were not used.

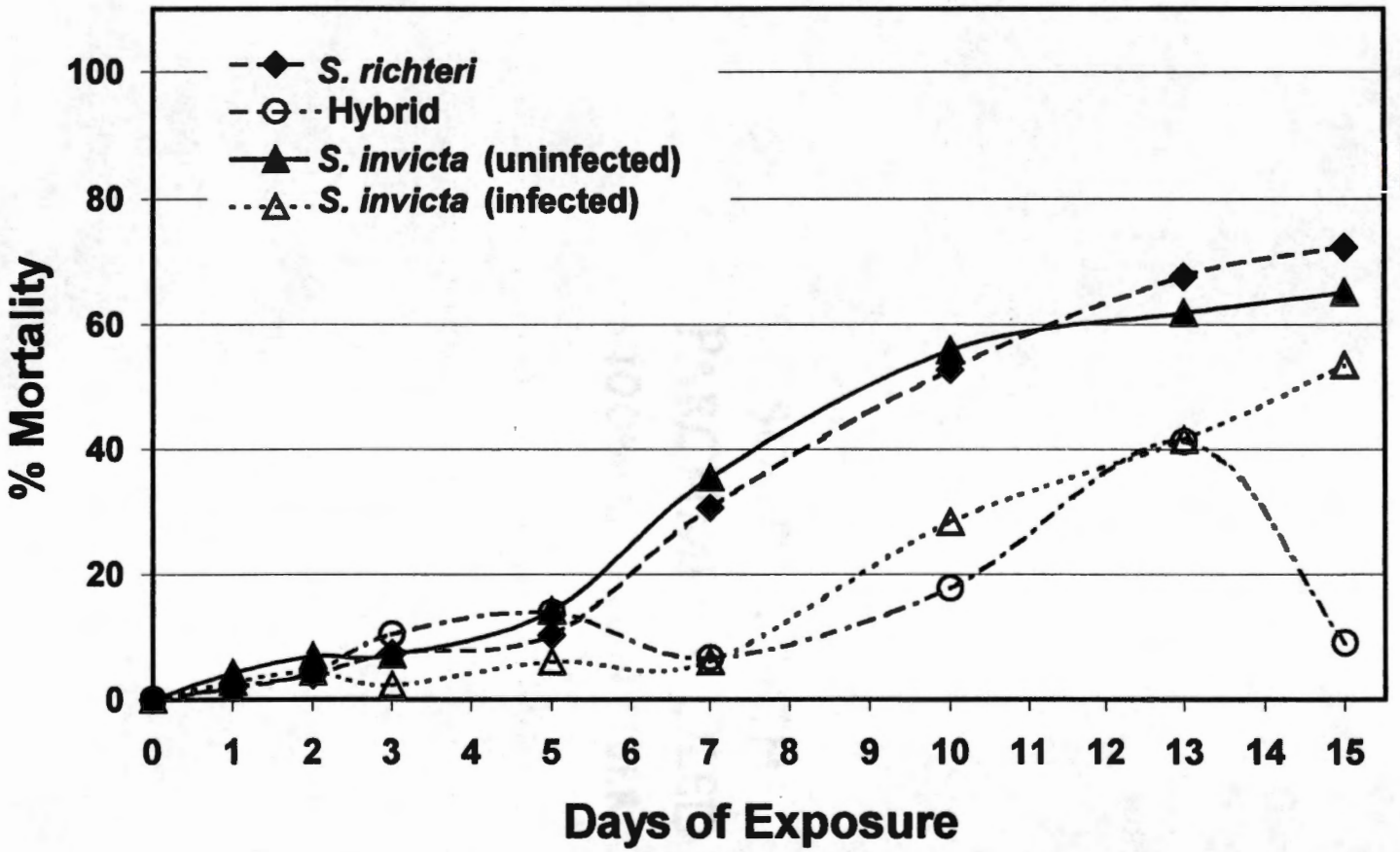


Figure 3.1. Mean percent mortality of spring-collected fire ant workers exposed to +4°C (positive regime) for up to 15 days. Ant samples were discarded after each day's determination of mortality; therefore, mortality determinations were independent for each sampling date. Data from the two tests were combined except for the last day (15) for the hybrid group for which there was only data from one test.

Although hybrid ants had not reached 50% mortality by the end of the test, infected *S. invicta* had 53.5% mortality at day fifteen. For *S. richteri* and uninfected *S. invicta* the estimated LD_{50} were 10. 5 and 11.0 days, respectively. Mortalities of 52.7% for *S. richteri* and 55.8% for uninfected *S. invicta* were observed on the tenth day of exposure at +4°C.

ii. Zero (+0.5°C) Temperature Regime

Significant ($P = 0.0175$; $df = 3, 36$; $F = 3.840$) differences in ant mortality were observed on the second day of exposure at the zero temperature regime (Fig. 3.2). On this day, uninfected *S. invicta* had 18.9% mortality compared to the low mortalities of *S. richteri* (5.1%) and the infected *S. invicta* (1.7%). After five days of exposure to this temperature regime, the uninfected *S. invicta* had a mortality of 61.5%, approximately double the mortality of the other three groups (hybrid = 23.1%, *S. richteri* = 27.5%, and infected *S. invicta* = 37.2%). Mortality was higher than 85% for all groups other than the hybrid (66.0%) by day ten. The hybrid ants had significantly ($P < 0.05$) lower mortality than the uninfected *S. invicta* from day five through the last day of exposure. On days seven and ten, hybrid ants also had significantly ($P < 0.05$) lower mortality than either the *S. richteri* or infected *S. invicta,* which were not significantly different from uninfected *S. invicta.* By day fifteen, mortality had reached 99.2% and 98.7% for the infected and uninfected *S. invicta*, respectively.

The uninfected *S. invicta* ants reached 50% mortality at some point between days 3 (29.1%) and 5 (61.5%), which seems to fit the LD_{50} of 4.0 days. At day five, *S. richteri* and infected *S. invicta* had 27.5% and 37.2% mortality, climbing to 68.1% and 73.6%,

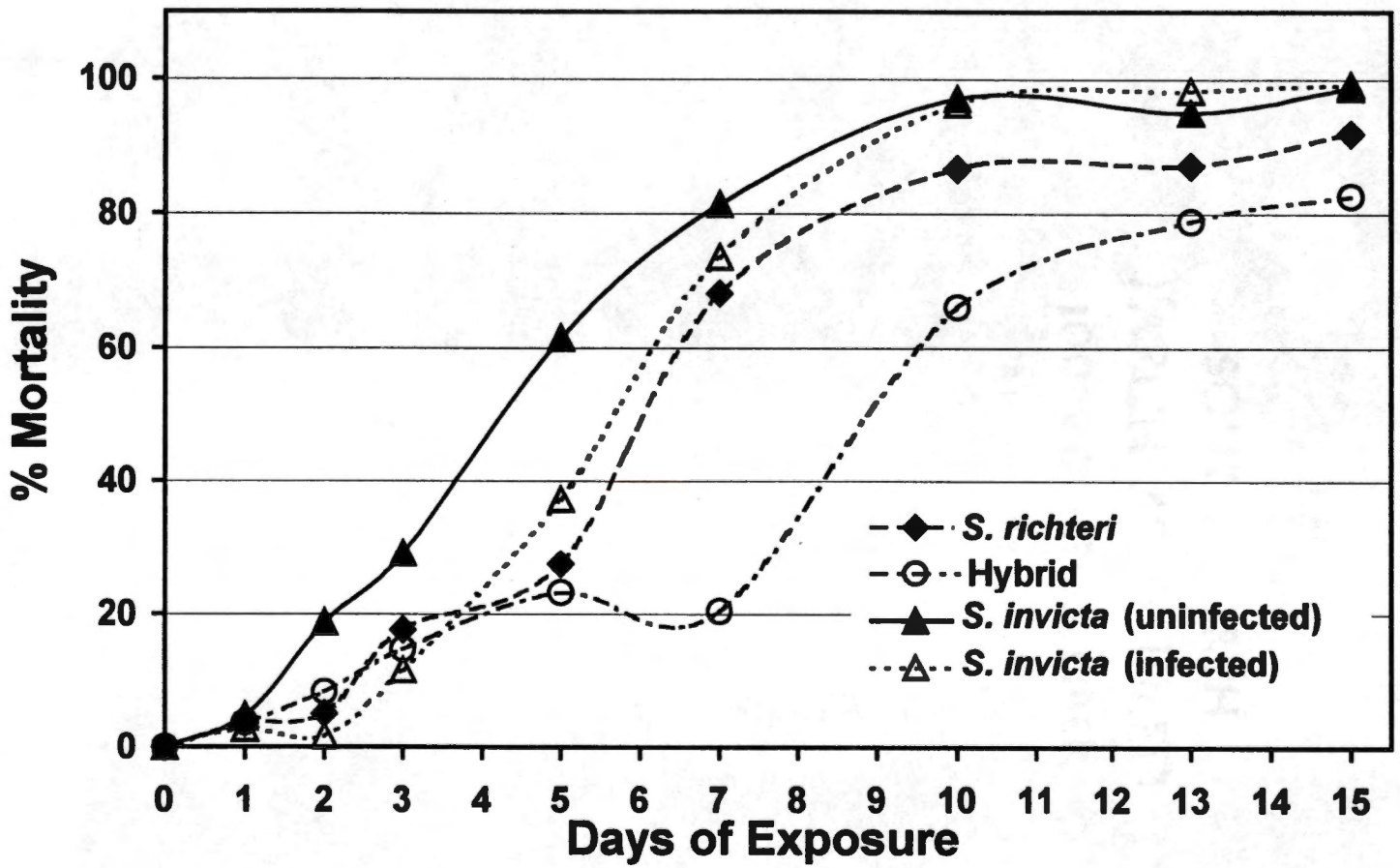


Figure 3.2. Mean percent mortality of spring-collected fire ant workers exposed to +0.5°C (zero regime) for up to 15 days. Ant samples were discarded after each day's determination of mortality; therefore, mortality determinations were independent for each sampling date. Data from two tests were combined except for the last day (15) for the hybrid group for which there was only data from one test.

respectively, by day seven. The LD_{50} for *S. richteri* was estimated at 5.7 days and for infected *S. invicta* at 5.2 days. Estimated LD_{50} for hybrid ants was 8.6 days; they had mortality rates of 20.4% on day seven and 66.0% on day ten.

iii. Negative (–4°C) Temperature Regime

High mortality was reached far faster at the negative temperature regime than at the other temperatures, as 100% mortality was reached by day seven in all four ant groups (Fig. 3.3). Mortality on the first day was low as all four ant groups had mortality less than 11%. By day three, the uninfected *S. invicta* displayed significantly ($P < 0.05$) higher mortality (82.1%) than all other groups (hybrid = 41.1%, *S. richteri* = 44.6%, and infected *S. invicta* = 65.6%). By day five, hybrid ants still had significantly ($P < 0.05$) lower mortality (88.0%) than both *S. invicta* groups (infected = 99.5% and uninfected = 100%).

Based on probit analysis, estimated LD_{50} values were > 2 but < 3 days for all ant groups. None of the groups reached 50% mortality by day two, but both *S. invicta* groups had surpassed it by day three. *S. richteri* and hybrid ants surpassed the 50% mortality mark sometime prior to day five but after day three.

C. Application of Ice-nucleating Bacteria

The two spray application trials yielded no significant (first trial $P > 0.05$ and second trial [$P = 0.9760$; $df = 2, 31$; $F = 0.024$]) differences among the water, Bti, and Psp treatments (Table 3.3). Comparisons between the first trial, which was applied

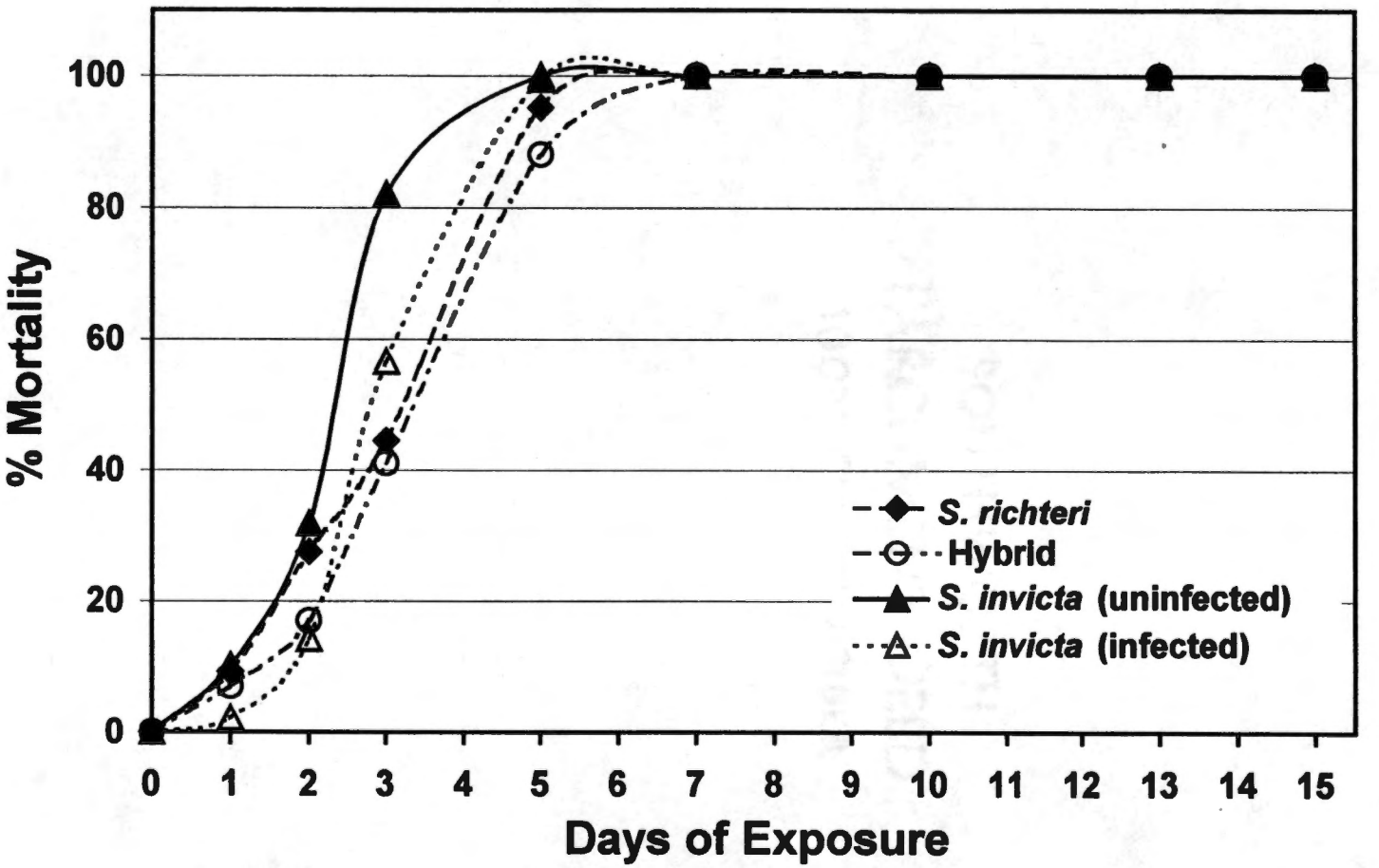


Figure 3.3. Mean percent mortality of spring-collected fire ant workers exposed to -4°C (negative regime) for up to 15 days. Ant samples were discarded after each day's determination of mortality; therefore, mortality determinations were independent for each sampling date. Data from the two tests were combined except for the last day (15) for the hybrid group for which there was only data from one test.

Table 3.3. Supercooling points (mean ± sem °C) of hybrid fire ants sprayed with bacterial suspensions or water, with or without Tween 80© and oil.

Suspension*	Distilled Sterilized Water	Distilled Sterilized Water with Tween 80© and Oil‡
Distilled Sterilized Water	-6.6 ± 0.11 a	-5.9 ± 0.13 b
Bacillus thuringiensis israelensis (Bti)	-6.2 ± 0.13 a	-6.0 ± 0.13 a
Pseudomonas syringae phaseolicola (Psp)	-6.3 ± 0.07 a	-6.0 ± 0.14 a

Means within the same column were not significantly different at the 0.05 level of confidence using Student-Newman-Keuls mean separation procedure.
Means within the same row followed by the same letter were not significantly different at the 0.05 level of confidence using Student-Newman-Keuls mean separation procedure.
* Approximately 50 ants were sprayed with 1 ml of either water or an opaque bacterial suspension.
‡ Tween 80© and vegetable oil were added to dissolve ant cuticular wax and aid in bacterial adhesion, respectively.

without oil and tween, and the second, with oil and tween added, showed ants sprayed with the water/oil/tween emulsion had significantly ($P = 0.0003$, $df = 1$, $F = 14.580$) higher supercooling points than those sprayed with pure water. Results for the submersion tests follow those of the spray applications, as no significance (first trial [$P = 0.9349$; $df = 2, 31$; $F = 0.067$] and second trial [$P = 0.6675$; $df = 2, 33$; $F = 0.409$]) was found in either trial. Ants subjected to the feeding test did not have supercooling points above the range of previously tested non-treated fire ants from that colony.

CHAPTER IV

Discussion

A. Supercooling

i. Thermocouple Size

Initially, cross comparisons of supercooling points between different sized ants were planned. However, the determination of difference in results obtained with the large and small thermocouples prevented size comparative analysis of the supercooling data. Two factors eliminated these comparisons: the significant difference between results of the same ant size group on the different size thermocouple, and the significant difference between ant size groups tested with large thermocouples when no difference existed with the small, more sensitive thermocouples. The significant difference also means that the supercooling results presented here cannot be accurately compared with those in previous fire ant supercooling publications (Table 4.1). These previous authors used larger thermocouples that would have been less accurate in recording the freezing points of insects as small as fire ants. Some comparisons may be possible when the thermocouples used were of the same size. Most researchers have used a 0.33-mm diameter microprobe (Diffie and Sheppard 1989, Francke et al. 1986, and Taber et al. 1987), but a 0.59-mm-diameter microprobe also has been used (Landry and Phillips 1996). With the $\geq$ 1.5°C drop in temperature between the measurements from the 0.01- and 0.1-mm thermocouples in the current experiments, temperatures read on 0.1-, 0.33-, and 0.59-mm

Table 4.1. Summary of supercooling points (°C) for imported fire ants and conditions under which results were obtained as determined by different researchers. *

Species	Researchers/date†	Small Ants	Large Ants	Season	Lab Time Prior to Testing
Solenopsis richteri	Diffie and Sheppard, 1989	–	–7.0	cold	Yes, unknown
	Francke et al., 1986	–6.3	–6.2	warm	Yes, a minimum of two weeks
Hybrid	Diffie and Sheppard, 1989	–	–5.9	cold	Yes, unknown
Solenopsis invicta	Francke et al., 1986	–9.4	–6.8	warm	Yes, a minimum of two weeks
	Taber et al., 1987	–4.5 to –5.9	–	cold	No, tested in field
	Diffie and Sheppard, 1989	–	–6.6	cold	Yes, unknown
	Landry and Phillips, 1996	–6.4‡		warm	Yes, one month

*Compare with current research results, see Table 3.1.
† Thermocouples with a diameter of 0.33-mm were used for all experiments except Landry and Phillips (1996) who used a 0.59-mm thermocouple.
‡ Ant size data combined for this supercooling point.

thermocouples cannot be considered analogous to each other or the true temperatures of the ants, but may allow examination of relationship differences among test groups.

ii. Colony Variation

Originally, supercooling tests were to take place as soon after collection as possible. Unfortunately, due to circumstances beyond control, the colonies collected prior to fall 1999 were maintained in the laboratory for a period of several months before testing. Results from this delayed testing supported the conclusion by Taber et al. (1987) that supercooling ability of imported fire ants undergoes change in the laboratory environment. However, Landry and Phillips (1996) found no difference among the ants they kept in the laboratory approximately one month before testing.

a. Tennessee Groups of Imported Fire Ants

The significant difference observed in the fall groups of ants was lost or reversed in the spring groups. The spring ants also were less uniform in supercooling point than the fall ants. Without either more data points throughout the seasons, or a better understanding of what causes changes in fire ant supercooling point, no conclusion can be reached on whether these differences are strictly due to seasonal variation within each colony or due to species. Previous supercooling studies were performed on ants collected and tested in either cold or warm weather conditions, but not both as reported here.

Only one other study compared supercooling points of *S. richteri* and hybrid ants (Diffie and Sheppard 1989). Those authors gathered results from large worker ants, collected under winter conditions, and housed in their nest soil in a greenhouse during testing. If the results for large fall *S. richteri* (–8.6°C) and hybrid (–8.2°C) of the present

study are compared with those in Diffie and Sheppard (1989) (–7.0°C and –5.9°C, respectively), it appears that during cold weather seasons, at least the *S. richteri* supercools to lower temperatures than hybrids. Furthermore, it appears that species acclimation may be occurring over time, because the ants tested here supercooled to lower temperatures than those in Diffie and Sheppard's study 10 years ago. However, the influence of different situations in ant housing and maintenance, as well as thermocouple size used for the tests, cannot be separated from the data.

Supercooling data was collected by Francke et al. (1986) on small and large *S. richteri* workers collected in Mississippi during spring and summer. Their mean supercooling points were –6.3°C for small and –6.2°C for large ants, compared with results herein for spring *S. richteri* small (–6.8°C) and large ants (–6.8°C). Both studies with ants collected during warmer weather resulted in small and large *S. richteri* having no significant difference in supercooling point. However, in the present study, small ants were measured using the small thermocouples, which assessed the supercooling points at temperatures more than a degree and a half higher than the large thermocouples. The ants used by Francke et al. (1986) also were housed in the dark at 22°C for at least two weeks prior to testing. The ants in the present study were housed under variable photoperiod and temperature conditions. Photoperiod was speculated as the cause for differences between results by Diffie and Sheppard (1989) and Francke et al. (1986), since the colonies maintained in a greenhouse were exposed to light conditions similar to the field. However, no further experimentation with photoperiod effect was conducted.

b. Florida Groups of Imported Fire Ants

Comparisons between ants collected during different seasons were not initially planned for *S. invicta* groups, as these ants were collected from Florida, where the warm climate allows fire ants to both forage and produce brood year round (Markin et al. 1974). However, extreme differences were observed between results of the winter and spring colonies of *S. invicta,* when no seasonal effect was expected. The winter *S. invicta* results were similar to those for preliminary groups maintained in the laboratory several months prior to testing. Thus, these differing results may be due solely to the winter colonies being shipped without queens or brood and having been collected a month prior to testing. Interestingly, this condition also may have affected the comparisons between the infected and uninfected ants. The apparent trend in winter ants of both sizes, in which *T. solenopsae*-infected ants supercooled to significantly lower temperatures than uninfected ants, was reversed in the spring *S. invicta* groups, which had brood and queen(s) and were tested within two weeks post collection. These results further demonstrate the possible effects of unnatural situations on fire ant supercooling.

Uninfected spring *S. invicta* appeared more cold hardy, i.e., had a lower supercooling point, than the infected ants. The expected result of uninfected ants appearing more cold hardy than the infected ants was further supported by analysis comparing the supercooling points of individuals with confirmed *T. solenopsae* infection and supercooling points from uninfected colonies. No significant difference occurred between the two groups' mean supercooling points in either large or small ants from spring ant collections. It is possible that those ants that could not be confirmed as infected, even though they were part of an infected colony, had an undetectable titer of

protozoan spores. Low spore count may be hard to detect with the methods used in this study. Failure to detect existing *T. solenopsae* infections may explain similar supercooling points for confirmed infected and apparently uninfected ants from infected colonies. However, similar supercooling points of ants from infected colonies, regardless of individual infection status, also suggest that *T. solenopsae* infection may have effects that are not limited to the infected individual but extend to all or most members of the colony. Since *T. solenopsae* infection has only recently been found in the United States, and no studies on fire ant supercooling have been conducted in South America, no other literature is available on supercooling points of *T. solenopsae*-infected colonies.

All four previous supercooling studies on fire ants tested *S. invicta* individuals that were presumed not infected with *T. solenopsae* as no mention of the protozoa was made in the reports and only a few locations in the United States have infected populations (Williams et al. 1998). Both Francke et al. (1986) and Landry and Phillips (1996) collected *S. invicta* in warm seasons and divided the ants into size groups. Results from the present study support those by Francke et al. (1986) (small = –9.4°C and large = –6.8°C) as the large *S. invicta* in the current study had a mean supercooling point of –6.8°C. Although in the present study different sized thermocouples were used for small and large ants, small *S. invicta* did supercool to colder temperatures than the large ants. Considering the differences observed between results of the two thermocouple sizes, if the small ants in this study had been tested on the larger thermocouple, as in the study by Francke et al. (1986), the mean supercooling point would have been a degree or more lower than the reported –7.6°C. Thus, the adjusted mean supercooling point of the small *S. invicta* would be closer to the –9.4°C mean reported by Francke et al. (1986).

However, as with previously mentioned studies, Francke et al. (1986) had different conditions for colony maintenance such as a 24 h-dark photoperiod. These different conditions may have influenced the supercooling results.

Results from Landry and Phillips (1996) cannot be compared with those presented here. These authors did separate ants by head capsule size, but results appear to have been combined, regardless of ant size, when no significant size/supercooling correlation was found. Landry and Phillips (1996) also used a thermocouple width much larger than other researchers and, hence, much less sensitive than thermocouples used in previous experiments. The ant colonies in their study also were maintained in the laboratory for a month prior to testing. Results presented by Taber et al. (1987) and in the current study indicate a strong possibility that fire ant supercooling points are affected by laboratory conditions.

Results of *S. invicta,* collected and tested in cold seasons, are presented by both Diffie and Sheppard (1989) and Taber et al. (1987). Unfortunately, despite use of identical equipment, differences in methodology make the validity of comparisons between the two studies uncertain. Taber et al. (1987) selected small workers and Diffie and Sheppard (1989) selected large workers as test subjects. Results found in the current study have demonstrated significant differences in the ability of a thermocouple, $\geq$ 0.1-mm diameter, to detect supercooling points from small and large sized ants. With a mean supercooling point of –6.6°C, the results of Diffie and Sheppard (1989) are similar to those of Francke et al. (1986) and the present study's large worker results (both =

–6.8°C). Taber et al. (1987) reported surprisingly warm (considering the large thermocouple used) supercooling points. The mean supercooling point, as reported by Taber et al. (1987), from field tests in February, was –4.5°C.

iii. Species Comparisons

Amongst previous supercooling point studies, only Diffie and Sheppard (1989) and Francke et al. (1986) compared multiple species of imported fire ant. Diffie and Sheppard (1989) found that supercooling points of large *S. richteri* workers were not significantly lower than those of *S. invicta,* and both had significantly lower supercooling points than hybrid ants. In the current research there was no significant difference between large *S. richteri* and *S. invicta,* but hybrid ants had a significantly lower supercooling mean than the two parent species. Francke et al. (1986) reported *S. invicta* large and small workers supercooled to lower temperatures than *S. richteri.* In the present study, small *S. invicta* supercooled to significantly lower temperatures than small *S. richteri*, but no significant differences in the larger ants were observed. Diffie and Sheppard (1989) reported use of the same test apparatus as Francke et al. (1986) had used earlier but collected and maintained their ants under different conditions.

iv. Head Capsule Measurements and Correlations

Smaller bodies of water, or in this case smaller ants, are expected to have lower supercooling points than larger bodies of water, or larger ants (Lee 1991). Yet, lack of a significant correlation between the head capsule width and the supercooling point was an interesting, but not completely unexpected phenomenon. Landry and Phillips (1996) not

only tested for a correlation between head capsule size and supercooling point, but also tested ant dry weight and supercooling point for correlation. No correlation was found for either set of values. Often, colonies of hybrid fire ants and *S. richteri* collected in Tennessee are composed of a higher proportion of large individuals than *S. invicta* colonies, as in the colonies of the experiments reported here. The separation and comparison of the supercooling data for only those ants with similar head capsule sizes simply reiterated the comparison results for the complete test groups. These results support the lack of correlation between ant size and supercooling point in imported fire ant populations.

B. Extended Low Temperature Exposure

Avoidance is usually the first line of defense in cold weather survival of insects (Denlinger and Lee 1998). It has been speculated that imported fire ants escape damaging cold temperatures by going deeper into their nests (Morrill 1977). Further, it is known that imported fire ants move within the column of the nest soil to find optimal temperatures (Pinson 1980) and may become trapped in the upper layers of the nest if a cold front moves in too rapidly (Diffie et al. 1997, Morrill et al. 1978, Green 1959). Test tubes used in the extended low temperature exposure offered the ants no structural protection from cold, such as a nest might provide. The ants succumbed to cold coma and were incapable of movement or escape within 24 h of placement in the refrigerators, thus limiting possible avoidance behaviors. Hence, the results from these tests are considered a measure of the physiological, not behavioral, cold hardiness of the tested ants.

No effects were observed on mortality rate due to the length of time that the ants were maintained under laboratory conditions, as had been observed with the supercooling point tests. Due to the length of the low temperature exposure tests and limits on space available, the shortest time span from initiation of the first test on a group to the end of the last test was 49 days, while the longest span was 92 days. The lack of significant differences between the two tests indicates that the time in the laboratory did not influence the results.

i. Positive (+ 4°C) Temperature Regime

Ant mortality, not surprisingly, was low throughout the first days of exposure to this temperature treatment. According to prior studies (Porter and Tschinkel 1993 and Calabi and Porter 1989), *S. invicta* derived benefits from allocating portions of the colony to cooler temperatures. Individuals at lower temperatures expressed lower metabolic and respiratory rates, allowing these individuals a 14% increase in life span of for every 2°C drop in temperature. This situation further benefits the colony as the decrease in metabolism of the colder individuals translates into a lower food demand and, hence, a lowered colony cost in food resources. However, without protective mechanisms, cellular function will eventually be affected by continued low temperature exposure. Irreversible cell damage through phase shifts of organelles and cellular fluids, as well as possible denaturing of enzymes, are suspected causes of insect mortality at above-freezing temperatures (Denlinger and Lee 1998). At the temperatures used in the extended exposure experiments, fire ants would have been at a low enough metabolic rate that the increase in mortality, seen in the uninfected *S. invicta* and the *S. richteri,* has to be

ascribed to actual physical damage initiated by the low temperatures and not to starvation.

The geographic locations of *S. richteri* infestations in both North and South America, and lack of previous evidence of hybrid advantage (Diffie et al. 1997 and Diffie and Sheppard 1989), lead to the expectation that, if any group showed an improved cold hardiness, it would be *S. richteri*. By day seven however, the mortality of *S. richteri* was not significantly different from the more tropically oriented *S. invicta,* and the hybrid displayed significantly lower mortality than either of the parent species. Also surprising was the low mortality rate of the *T. solenopsae*-infected *S. invicta*. Infection with a debilitating disease was expected to decrease survival under unfavorable temperatures, but at the +4°C temperature regime it actually imparted resistance. At this point, it is unknown if the microsporidial infection produces or promotes production of protecting chemicals or some other protective mechanism occurs.

ii. Zero (+ 0.5°C) Temperature Regime

The +0.5°C treatment is the first regime that showed *S. invicta* to have a significantly lower cold injury resistance than the other three groups. *S. richteri* and hybrid ants had mortality rates less than half that of *S. invicta,* which had already surpassed 50% by the fifth day of exposure. By the seventh day of exposure, the *T. solenopsae*-infected group had succumbed to cold injury and no longer was significantly more cold hardy than the uninfected *S. invicta. S. richteri* still showed little or no advantage over the two groups of *S. invicta*. The hybrid, however, maintained low

mortality through most of the test period, indicating a hybrid advantage in colder climates.

Recently published winter mortality data, from a three-year field study of an isolated population of *S. invicta* in east Tennessee (Callcott et al. 2000), support results displaying a relationship between high mortality in *S. invicta* and extended low temperature exposure. This *S. invicta* population apparently is no longer present in Tennessee. The authors examined the relationship between two variables, percentage of change in number of spring colonies and percentage of change in number of colonies since the previous fall, and several climatic parameters. These parameters included: the lowest mean monthly maximum air temperature during winter, the lowest mean monthly minimum air temperature during winter, the number of consecutive days with the maximum air temperature below 0°C, and the number of consecutive days with the maximum air temperature ≤ 1.1°C. Their results indicated a high positive correlation between winter mortality and number of consecutive days with maximum temperature ≤ 1.1°C.

Highest mortality (87.5%) was in the winter of 1993-94, which had seven consecutive days at ≤ 1.1°C. Coincidentally in the present study, 81.4% mortality of uninfected *S. invicta* was observed after seven days at the +0.5°C regime. According to Callcott et al. (2000), the winter of 1995-96 caused a 78.9% reduction in *S. invicta* colonies after a five-day exposure to temperatures ≤ 1.1°C. If 1.1°C was the maximum for these days, at night it was most likely colder, allowing lower temperatures to penetrate the mound.

Though Callcott et al. (2000) did not report cloud cover for the days that had a maximum of 1.1°C, it would be expected that cloudy days, as are often experienced in winter, would prevent sufficient insolation of the mound, thus leaving the mound at low temperatures for consecutive days, similar to treatment conditions. Sunny days would allow nest soil to warm up providing the ants with a warmer nest. Therefore, it could be expected that ants in a natural setting exposed to low temperatures only briefly, because of nest soil reheating with solar exposure, would not suffer high mortality. The ants counted in the first days of low temperature exposure tests did not have high mortality and would be considered to have been only under a brief exposure.

iii. Negative (– 4°C) Temperature Regime

Within seven days of exposure to – 4°C, 100% mortality was observed for all ant groups. For the short period before day 7, uninfected *S. invicta* was more susceptible to cold injury than the other groups, and hybrid ants were less susceptible. These findings support the theory that the distribution of *S. richteri, S. invicta,* and their hybrid is linked to cold hardiness. This temperature regime was close to the supercooling point of all ant groups tested, and extended exposure may have caused ice formation in their hemolymph above recorded supercooling point temperatures. The weather in the current imported fire ant range probably does not often produce temperatures this low in the mound for as long as in the experiment, but the further north the fire ant range expands, the more likely fire ants will encounter extended periods of temperatures below 0°C.

C. Ice-nucleating Bacteria

i. Topical Applications

The use of external contact with ice to initiate freezing at temperatures above the supercooling point has been recorded for several freeze-tolerant insects. It was thus expected, especially in light of successful reports from Landry and Phillips (1996), that external contact with the ice-nucleating bacteria would have a significant effect on the supercooling point of imported fire ants. However, imported fire ants have been reported to have several well-developed hygienic behaviors that deter prolonged exposure to potential pathogens (Oi and Pereira 1993). After the topical application treatments, regardless of whether application was by spray or submergence, extensive grooming behavior was observed in all live individuals. Thus, it is likely that the tested ants had effectively removed enough of the ice-nucleating bacteria to prevent ice formation at higher temperatures. Results presented in the study by Landry and Phillips (1996) were obtained after the test groups were sprayed with suspensions daily for five consecutive days and then tested immediately after application on the fifth day. This suggests that an accumulation of sprays was required to either provide enough ice-nucleating bacteria on the ants, perhaps in joints and other areas the ants could not clean, or possibly reduce efficiency of grooming behavior as ants became accustomed to the spray routine.

ii. Feeding Application

Though the ants offered the bacterial/honey water suspension appeared to consume the fluid, ingestion of the bacteria is not certain. Adult imported fire ants are

known to have a structure that sieves out particles ≥0.88μm in diameter (Glancey et al. 1981). This structure, however, is made of setae and occasionally allows passage of larger particles. Thus, it was anticipated that the ants in this trial either would be able to ingest some of the bacteria they were given, or that enough might remain in their infrabuccal cavity to initiate freezing at higher temperatures. It was noted after this trial was terminated, that a small pile of unidentified white material had been gathered or deposited by the ants on the substrate of the nest cell. This pile may have been composed of ejected buccal pellets formed from bacteria sieved out of ingested honey water.

CHAPTER V

Conclusions

Based on the mild temperate to tropical climates from which *S. richteri* and *S. invicta* originate, they would have had little need to develop long-term cold survival mechanisms. Thus, it was unexpected, especially for the more tropical *S. invicta,* that they would infest the colder territories that they presently have in the United States. The construction of the mound provides a warmer environment than found either on the surface or within compact soil, if the mound is heated by sunlight (Pinson 1980). Also, the ants have been shown to be sensitive to temperature change and seek optimal temperatures in laboratory settings (Cokendolpher and Francke 1985, Cokendolpher and Phillips 1990, Porter and Tschinkel 1987, Porter and Tschinkel 1993) or within the mound (Pinson 1980). Additionally, imported fire ants usually cease activities outside the nest well before risk of cold coma (Markin et al. 1974 and Porter and Tschinkel 1987). The observed low mortality in the first few days of exposure to low temperatures indicates that *S. richteri, S. richteri* x *S. invicta* hybrid, and *S. invicta* (regardless of *T. solenopsae* infection) are capable of recovery from low temperatures above their freezing points, if the exposure is not prolonged. Regular daily insolation of the mound is likely to warm the nest soil and keep the ants from exposure to consistently cold temperatures. Although low mortality was observed among ants exposed to moderately cold temperatures, surviving ants were not tested for continued capacity as functional members of their colonies. Further research, specifically with intermittent exposure to low temperatures and observations on the functional ability of cold-exposed ants may

provide evidence on the effects of situations likely to be encountered by colonies in the field.

The combination of a warming mound, avoidance of cold temperatures, and possible ease of recovery from short-term low temperature exposures could explain the unexpected extremes of imported fire ant range within the United States. However, the abundance of hybrid colonies and polygyne *S. invicta*, both of which are rarely found in their native range, in addition to desiccation resistant populations found in Texas, now demonstrate that fire ants can change and adapt reasonably well to new conditions.

Several authors have attempted to gain insight into fire ant ability to survive cold weather through the use of supercooling point information. Because fire ants are not freeze tolerant, it was assumed that lowered supercooling points might be the mechanism used to avoid freeze damage and survive cold situations. However, based on the current study and that by Taber et al. (1987), it appears that supercooling points of imported fire ants can be affected by removal from field conditions and the time spent under unnatural conditions in the laboratory. Furthermore, tests with two sizes of thermocouples revealed the significance of thermocouple size in fire ant testing.

The present study did not consider the ability of different castes and life stages to survive low temperatures. *S. invicta* worker pupae and larvae were found in a previous study to supercool to significantly lower temperatures (–21.4 and –12.0°C, respectively) than adults (Francke et al. 1986). Pupae and larvae do not, however, survive extended periods without adult attendants, and it is unknown if proper development continues even if these immature stages do not freeze. The lower supercooling points may provide some

short-term protection from fast-moving cold fronts that could immobilize workers tending brood at more exposed nest levels.

Attempts to measure acclimation ability through supercooling point have produced conflicting results. Colonies maintained at different temperature regimes in the laboratory prior to supercooling point determination were not significantly different (Francke et al. 1986, Landry and Phillips 1996). Supercooling tests performed in the field every two weeks over an 18-wk period found a significant difference among samples taken throughout the season (Taber et al. 1987), but the authors hesitated to attribute this to acclimation. The present study showed differences between fall and spring results, but without more test dates these differences cannot be attributed to seasonal acclimation. Furthermore, the small amount of change observed in supercooling point argues against it as a seasonally improved (acclimated) mechanism to escape cold injury. Also, considering the small body volume of imported fire ants, their supercooling ability may be a function of their small size.

When protected in their nests within their current range, imported fire ants would rarely be exposed to temperatures close to their supercooling points. Even if higher supercooling points resulted from application of ice-nucleating bacteria (Landry and Phillips 1996), without an accurate measurement of the ants' supercooling temperatures, it cannot be determined whether these warmer supercooling points would be reached within the nest soil.

Beyond the question of exposure to sufficiently low temperatures, possible monetary and time expenditures would render impractical the field use of the current application method for ice-nucleating bacteria. The significant differences produced in

the experiments by Landry and Phillips (1996) were a result of five days of direct spray application, whereas, single spray and submergence applications used in the present study produced no significant difference between the treatments. Also, some of the more effective ice-nucleators, such as *P. syringae,* are considered plant pathogens, and their use in large amounts in the field would not be desirable.

The feeding trial attempted to circumvent possible problems of spray application, as a bait-formulation could be distributed throughout the colony given sufficient time. The workers tested in the feeding trial were without brood and thus without any fourth instar larvae that might have been better able to consume the bacteria. Future testing of ant groups with larvae may reveal significantly different results. Further study in application of ice-nucleating bacteria is needed to transform it into a viable fire ant control option. Until then, it may be more efficient to drench the mounds with water immediately before exposure to freezing temperatures or prior to nightfall during cold weather. This action would produce a fire ant-freezing situation similar to that reported by Green (1959) when rain-soaked mounds froze, killing the trapped ants.

Unlike freezing, exposure to occasional extended periods of low temperature may frequently occur in the colder regions in the imported fire ant range. Both the field experiments by Callcott et al. (2000) and the laboratory experiments in this research have shown this kind of exposure to be lethal after a certain number of days. Whether a nest receives sufficient insolation to re-warm the ants may be critical in fire ant winter survival as indicated by accounts of inability to colonize densely wooded mountains in northeastern Georgia (Diffie et al. 1997) and inactivity of mounds located in areas shaded by pine trees (Morrill et al. 1978). Heavily wooded sites, such as parks and wildlife

refuges, may help limit fire ant infestation by providing shaded areas that maintain low temperatures.

Results from laboratory-induced low temperature exposure and field reported results by Callcott et al. (2000) suggest that future field studies may benefit from monitoring temperatures within the nest. Nest monitoring would allow determination of how insolation affects temperatures within a fire ant mound and ant survival during cold weather. Further laboratory studies using different temperature regimes and longer test periods also could provide a more precise account of ant mortality under exposure to extended periods of low temperature. This information may be useful in predicting the likelihood of fire ant colonization as attempted by Wiley et al. (2000), or in degree-day prediction models for severity of fire ant re-infestation pressures and activity levels in spring. Fire ant-predictive modeling would further benefit from studies focusing on *S. richteri* and the *S. richteri* x *invicta* hybrid, since current models have been primarily based on *S. invicta* information.

The *S. richteri* x *invicta* hybrid had significantly lower mortality than *S. invicta* when exposed to low temperatures in the current research. This difference in survival supports the hypothesis that lower tolerance of cold weather conditions influences the geographical distribution of imported fire ant species. Furthermore, though part of the region assumed to be *S. invicta*-infested in the Carolinas is located at more northern latitudes than the *S. richteri* and hybrid infestation zones, the *S. invicta* are in milder coastal regions. *S. richteri* and its hybrid are more continental and generally subjected to harsher winters. If hybrid ants in the field survive for longer periods at low temperatures as observed in the laboratory, they would have an advantage over the less cold-tolerant *S.*

invicta. Also, the low temperature exposure tests reported herein used fire ants collected in warm spring weather. For *S. richteri* and hybrid ants collected in spring, hybrid workers had equal or better supercooling ability to that of the *S. richteri*, whereas results for fall-collected ants suggested the opposite. Hence, conducting extended exposure experiments using ants collected in other seasons may yield different results.

Field research on winter survival of Georgia populations of *S. invicta* and its hybrid was inconclusive. Mortality of hybrid colonies in that study was attributed to a severe cold front that moved through the hybrid ant site but not the *S. invicta* sites (Diffie et al. 1997). Microclimate variation experienced at different field locations can determine colony survival. Measurement of climatic variables affecting individual nests may be necessary to ensure proper interpretation of field data.

Results of the extended exposure experiments were obtained without allowing ants the shelter of their nests. This implies that hybrid ant advantage over *S. invicta* may be expressed at the cellular level and not be behavioral. Further exploration into the cellular-level advantages of hybrid ants or disadvantages of *S. invicta* may prove fruitful in elucidating observed differences in cold tolerance. If superior survival of hybrid colonies is not supported by field tests, a closer look at fire ant behavior may provide clues to disparity between laboratory- and field-generated results.

The apparent resistance to cold injury at the +4°C regime of the *T. solenopsae*-infected ants in this study indicates a possible survival advantage at colder climates for infected individuals. This situation is of some importance with respect to the success of fire ant biological control efforts utilizing *T. solenopsae*. If infected colonies have enough of an advantage over uninfected, they may out compete them, which may lead to an

increased spread of infection in colder regions. It also might allow further northern penetration by *S. invicta* when infected. Further study is needed to determine how infection improves the ability of *S. invicta* to tolerate low temperatures, or if there are other unobserved factors determining these results.

Distributions of *S. richteri* relative to *S. invicta* in both the native and introduced ranges suggest that *S. richteri* possess an advantage in cold tolerance. This hypothesis has not been supported in either the supercooling point or extended exposure experiments. Consequently, field studies, acclimation tests, close examination and comparison of behavior, and more extended low temperature exposure tests would be beneficial in exploring this hypothesis. Also, the size difference of workers in *S. richteri* and hybrid colonies when compared to *S. invicta* workers may be of importance to their cold weather survival. Head capsule measurements presented here support the general observation that *S. invicta* colonies are composed of smaller workers. Results from temperature studies on *S. invicta* developmental rates determined brood reared at lower temperatures had longer developmental times and thus emerged as larger adults (Porter 1988). The larger size of *S. richteri* and hybrid ants may be the result of brood reared in cooler climates causing slower developmental rates. Also, these ants may select cooler temperatures within the mound than *S. invicta* and thus rear generally larger workers to improve colony survival through increased longevity and lower calorie/mg of ant cost (Calabi and Porter 1989).

In summary, how imported fire ants and their hybrid survive cold weather is a complex subject that researchers have only begun to explore. Most fire ant research has been conducted with *S. invicta.* The assumptions that *S. richteri* and the hybrid are similar to *S. invicta* behaviorally and physiologically in their responses to low

temperatures may be unfounded. Furthermore, the studies including *S. richteri* and the hybrid have focused on supercooling, practically ignoring other avenues of cold tolerance testing. Supercooling ability results presented herein showed little similarity with those of extended low temperature survival and did little to support the geographic distribution of imported fire ants. The results herein for *S. invicta,* the geographic distribution of the species, and the recent winter mortality study by Callcott et al. (2000) suggest it is necessary to study cold tolerance parameters beyond supercooling.

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APPENDICES

APPENDIX A. Infection Rates and Experimental Uses of *S. invicta* Colonies Infected with *Thelohania solenopsae.*

Colony		% Infection Rate*	Experimental Use
Spring	#1	70	Supercooling point determination and both trials of the extended low temperature exposure
	#2	80	Supercooling point determination and both trials of the extended low temperature exposure
	#3	70	Supercooling point determination and the second trial of the extended low temperature exposure
	#4	73.1	Supercooling point determination and the first trial of the extended low temperature exposure
	#5	46.7	Supercooling point determination and both trials of the extended low temperature exposure
	#7	70	Both trials of the extended low temperature exposure
Winter	#1	85	Supercooling point determination
	#2	20	Supercooling point determination
	#3	70	Supercooling point determination
	#4	65	Supercooling point determination
	#5	55	Supercooling point determination

* Infection rates were determined from ants used in experiments. Ten small workers, ten large workers, and when available up to 10 female alates were used in infection rate determination.

APPENDIX B. Colony Mean Supercooling Points (mean ± sem) for Small and Large Imported Fire Ant Workers Throughout All Periods of Testing.

Species *	Collected	Tested‡	Colony	Supercooling Point	
				Small Ants	Large Ants
S. richteri	Spring and Summer 1999	Fall 1999	1	9.0 ± 0.75	-
			2	10.1 ± 0.46	10.7 ± 0.50
			3	12.8 ± 0.76	14.4 ± 0.57
			4	7.2 ± 0.22	-
			5	6.8 ± 0.17	-
	Fall 1999	1 wk	1	6.8 ± 0.12	8.7 ± 0.26
			2	6.7 ± 0.11	8.7 ± 0.23
			3	6.9 ± 0.10	8.1 ± 0.27
			4	6.6 ± 0.13	8.7 ± 0.24
			5	7.0 ± 0.24	8.8 ± 0.19
		3 mo	1	7.3 ± 0.23	8.1 ± 0.24
			2	6.6 ± 0.15	7.1 ± 0.23
			3	7.9 ± 0.33	8.1 ± 0.34
			4	6.5 ± 0.21	8.8 ± 0.59
			5	7.0 ± 0.32	7.9 ± 0.18
	Spring 2000	1 wk	1	6.4 ± 0.10	6.7 ± 0.21
			2	7.8 ± 0.51	7.0 ± 0.17
			3	6.5 ± 0.11	6.8 ± 0.09
			4	7.0 ± 0.17	6.7 ± 0.14
			5	6.2 ± 0.07	6.7 ± 0.22
Hybrid	Spring and Summer 1999	Fall 1999	1	7.2 ± 0.22	8.8 ± 0.20
			2	11.6 ± 0.56	9.1 ± 0.29
			3	6.4 ± 0.09	8.3 ± 0.25
			4	7.6 ± 0.24	9.5 ± 0.25
			5	7.6 ± 0.66	8.9 ± 0.18
	Fall 1999	1 wk	1	5.9 ± 0.13	7.9 ± 0.22
			2	6.3 ± 0.09	8.0 ± 0.24
			3	6.1 ± 0.14	8.5 ± 0.21
			4	6.3 ± 0.10	8.4 ± 0.17
			5	6.3 ± 0.10	8.3 ± 0.24
		3 mo	1	6.4 ± 0.18	7.3 ± 0.15
			2	6.6 ± 0.12	7.6 ± 0.15
			3	7.1 ± 0.14	8.4 ± 0.18
			4	6.0 ± 0.12	7.1 ± 0.14
			5	6.3 ± 0.13	7.1 ± 0.13

APPENDIX B. Continued

Species	Collected	Tested	Colony	Supercooling Point	
				Small Ants	Large Ants
Hybrid	Spring 2000	1 wk	1	6.4 ± 0.12	7.2 ± 0.24
			2	6.4 ± 0.11	6.8 ± 0.13
			3	7.2 ± 0.25	8.9 ± 1.05
			4	6.6 ± 0.12	7.5 ± 0.27
			5	7.7 ± 1.00	6.9 ± 0.27
S. invicta (uninfected)	Winter 1999	1 mo	1	8.6 ± 0.25	8.4 ± 0.33
			2	7.5 ± 0.15	7.4 ±0.10
			3	8.1 ± 0.14	7.5 ± 0.07
			4	9.5 ± 1.12	7.3 ± 0.25
			5	10.1 ± 1.24	6.9 ± 0.14
	Spring 2000	2 wk	1	7.0 ± 0.10	7.9 ± 0.38
			2	7.5 ± 0.45	6.1 ± 0.21
			3	6.8 ± 0.28	6.1 ± 0.14
			4	7.6 ± 0.24	6.5 ± 0.11
			5	9.1 ± 0.86	7.3 ± 0.37
S. invicta (infected)	Spring and Summer 1999	Fall 1999	1	9.9 ± 0.56	6.9 ± 0.15
			2	11.0 ± 0.31	8.2 ± 0.76
			3	12.8 ± 0.49	12.1 ± 0.71
			4	11.6 ± 0.81	12.1 ± 1.00
			5	6.5 ± 0.38	5.5 ± 0.07
	Winter 1999	1 mo	1	7.9 ± 0.35	8.0 ± 0.64
			2	15.5 ± 1.28	7.9 ± 0.35
			3	12.5 ± 1.68	8.2 ± 0.29
			4	5.9 ± 0.12	6.2 ± 0.12
			5	16.7 ± 1.42	11.0 ± 1.27
	Spring 2000	2 wk	1	7.5 ± 0.49	6.3 ± 0.10
			2	6.2 ± 0.14	6.3 ± 0.10
			3	6.3 ± 0.20	6.2 ± 0.09
			4	6.3 ± 0.13	6.3 ± 0.08
			5	6.0 ± 0.06	5.8 ± 0.09

* Colonies of *S. invicta* were classified as either infected with *Thelohania solenopsae* or uninfected.

‡ The time period after collection in which testing occurred.

VITA

Shannon Suzanne James graduated cum laude in May of 1995, with a B. S. in biology, from Mississippi University for Women. During the time she spent in Mississippi she became acquainted with the imported fire ant and was inspired to research their control. In January of 1999, she accepted a graduate research assistantship and enrolled at the University of Tennessee in the Department of Entomology and Plant Pathology under the guidance of Drs. Pereira and Vail.

Shannon has presented her research at entomological meetings throughout the duration of her term with the Department of Entomology and Plant Pathology, and was awarded honorable mention for her poster presentation at the Entomological Society of America annual meeting in Montreal on December 2000. Shannon is ecstatic about embarking upon her entomological career and plans to maintain her membership with the Entomological Society of America.