Entomopathogenic Nematode Management of Small Hive Beetles (*Aethina tumida*) in Three Native Alabama Soils Under Low Moisture Conditions

by

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Abstract

The overall goal of this work was to determine the efficacy of entomopathogenic nematodes (EPNs) on *Aethina tumida* Murray (Coleoptera:Nitidulidae) small hive beetle (SHB) in different soil types under low moisture conditions to improve current integrated pest management practices. The objectives were to 1) determine the pupation success of SHB wandering larvae in natural non-autoclaved and sterile autoclaved soil; 2) determine the efficacy of EPNs on SHB wandering larvae in natural non-autoclaved and autoclaved soil in low moisture conditions; and 3) determine the efficacy of EPNs on SHB wandering larvae in three natural non-autoclaved soil types at low moisture levels. The Alabama soils we tested were Kalmia loamy sand (KLS), Benndale fine sandy loam (BFSL), and Decatur silt loam (DSL). For this work, commercially purchased *Heterorhabditis bacteriophora* Poinar, *Steinernema feltiae* Filipjev, and *Steinernema kraussei* Steiner, as well as commercially purchased and laboratory reared *Heterorhabditis indica* Poinar, Karunaka & David, *Steinernema carpocapsae* Weiser, and *Steinernema riobrave* Cabanillas, Poinar & Raulston were tested. We evaluated population densities of 5%, 10%, 20%, 40%, and 80% third stage infective EPN juveniles (IJ3) per 130cc soil. In objective one, pupation success in SHB population densities of 5, 10, and 20 wandering larvae per Petri dish in KLS were similar. Thus, for objectives two and three we used a SHB population density of 5 SHB wandering larvae per Petri dish. Objective two evaluated six commercially purchased and laboratory reared EPN species in natural non-autoclaved and autoclaved KLS soil. Of the six commercially purchased species, *S. carpocapsae* achieved the highest efficacy across all EPN population densities and in both natural non-autoclaved and autoclaved soil with the 69.4% and 84.1% efficacy, respectively. *Steinernema riobrave* and *H. indica* achieved the next highest efficacies, however, they were significantly less effective that *S. carpocapsae.* Of the laboratory reared EPNs, the highest efficacy

for *S. carpocapsae*, *S. riobrave,* and *H. indica* was achieved at the population density of 80% IJ3 per 130cc soil. *Steinernema carpocapsae* parasitized 86.7% SHB wandering larvae across all population densities tested. The third objective included all three soil types at the moisture content of 50% field capacity for each soil. At the highest population density, *S. carpocapsae* achieved the best efficacy in KLS, BFSL, and DSL soils at 94.0%, 80.0%, and 47.0%, respectively. In all low moisture EPN experiments, efficacy of each EPN species on SHB wandering larvae was improved when higher EPN population densities were applied. In conclusion, this work suggests that *S. carpocapsae* could be a promising biological control agent to implement into an integrated pest management system for control of SHB in Alabama during low moisture conditions.

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Chapter 1: Review of Literature

Statement of Purpose

Apis mellifera, European honey bees, are important pollinators that support crop growth and environmental health globally. Pests, including *Aethina tumida*, small hive beetles (SHB), can negatively affect colony health and productivity, subsequently limiting the pollination potential of *A. mellifera*. Integrated Pest Management (IPM) systems are currently the best control options for SHB because chemical controls negatively affect *A. mellifera* and colony products. Recent studies suggest that entomopathogenic nematodes (EPN) that feed and reproduce on SHB may represent an efficient biological control option for SHB IPM. To date, there is little knowledge about how EPNs perform under field-realistic conditions such as the effects of soil texture and classification. The main objective of this research is to determine the efficacy of six entomopathogenic nematodes for controlling SHB larva and pupa in three different soil types found in Alabama in order to improve current IPM practices.

Apis mellifera the honey bee

The order Hymenoptera (Linnaeus, 1758), suborder Apocrita (Rasnitsyn, 1975), and superfamily Apoidae (Michener, 1965) classifies an estimated 20,000 solitary, social, and eusocial bee species globally (Michener, 1969, 1974; Duffield et al., 1984). Solitary bees make up approximately 85% of the known bee species, while social and eusocial bees make up only 15% (Batra, 1984). Solitary bees are important for pollination; however, they are difficult to manage unlike social and eusocial bees (Batra, 1984). Of the estimated 20,000 global bee species, only about 3,500 are found throughout North America (Southwick and Southwick, 1992; Richards and Kevan, 2002). Estimations are difficult to determine due to scarce field observations caused by the relatively large spatial abundance of wild, solitary bee populations

(Michener, 1974; Koh et al., 2016). In Alabama, there are currently no definitive data on numbers of solitary, social, and eusocial bee species. The majority of current bee knowledge and research favors bees that are manageable and economically important such as the genus *Bombus* (Latreille, 1802) and *Apis* (Linnaeus, 1758).

According to Day (1979), *A. mellifera* (Linnaeus, 1758) are in the class Insecta, order Hymenoptera, suborder Apocrita, superfamily Apoidea, family Apidae, tribe Apini, and genus *Apis.* There is conflicting data regarding variation within the genus *Apis.* Ashmead (1899) attempted to consolidate previous classification work on the family Apidae conducted by over seven scientists and concluded that there are two subfamilies of Apidae and three or four genera under those subfamilies depending on which scientist is cited. Dadant (1918) described only two races of bees, the common black bee or the Italian bee. Rutter (1896) also compared previous research on variability within the genus *Apis* based on geography and morphological measurements. He found that the genus *Apis* has had up to 600 named species and, of those, there are only four that can be considered species: *A. floria* (Fabricius, 1787), *A. cerana* (Fabricius, 1794), *A. mellifera,* and *A. dorsata* (Fabricius, 1794). Rutter (1896) then describes 23 races of *A. mellifera.* Ruttner (1988) outlined four definitive species of *Apis,* again, while simultaneously mentioning that his descriptions may actually include five or six species total. Engel (1999) as well as Arias and Sheppard (2005) recognize 10 species of *Apis* and three subspecies of *A. mellifera.* In 2015, an online dichotomous key of the species within *Apis* mentions four definitive species as well as a fifth species that may be recognized in some literature (Pauly and Hadel, 2015 http://www.atlashymenoptera.net/page.asp?id=238). The same dichotomous key also lists 30 subspecies, also referred to as races, of *A. mellifera.* Mortensen et al. (2013) identifies only 20 subspecies of *A. mellifera*. Disagreement regarding the diversity of subspecies or races of *A. mellifera* are a result of a multitude of tools available for classification

and a lack of standardization of such tools. These tools include classification based on behavioral, morphological, and molecular characteristics as well as phylogenetic and genetic information available today (Meixner et al., 2013). Of the *Apis* spp., *A. mellifera,* the European honey bee, is the most studied insect pollinator in the world due to their pollinator potential and the phenomenon's surrounding the decrease in global colony numbers (Otis, 1991; Bekić et al., 2014). According to the Bee Informed annual colony loss surveys, total colony loss in North America in 2010 and 2019 were approximately 27% and 45% (Bruckner et al., 2019). Multiple factors contribute to *A. mellifera* colony loss such as poor management, chemical contamination, and natural pests (Mullin et al., 2010; Zhu et al., 2014; Krupke and Long, 2015).

Distribution and economic importance of *Apis mellifera*

Apis mellifera are native to Europe, Africa, and the Middle East; however, global popularity has allowed this species to spread everywhere except Antarctica (Mortensen et al., 2013) via human trade due to their pollination potential (Han et al., 2012; Smith et al., 2013; Hung et al., 2018). The use of pollinating insects in agriculture alone is a billion dollar industry (Smith et al., 2013). As global human population continues to increase, *A. mellifera* pollination services have become a vital tool in feeding the growing populations. Mortensen et al. (2013) estimates that *A. mellifera* are responsible for more than 30% of the food we eat globally and are credited with $15 billion dollars in crops to the United States annually. Almond, apple, melons, alfalfa seed, plum, avocado, blueberry, cherry, pear, cucumber, sunflower, cranberry, kiwi, coffee, grapefruit, oilseed rape, onion, passion fruit, peach, pigeon pea, pumpkin, red clover, strawberry, and buckwheat are some of the most common crop systems that use managed *A. mellifera* colonies to increase yield potential (Morse and Calderone, 2000; Hodges et al., 2001; Calderone, 2012).

Along with their pollinator abilities, *A. mellifera* colonies are profitable for the products they produce. Almost every product that comes from a hive can be used or sold, including the bees themselves (Hodges et al., 2001). In the United States these products include bulk honey, retail packaged honey, comb honey, beeswax, pollen, live queens, developing queen cells, packaged bees, nucs, complete hives, bee brood as a food source, venom, royal jelly, and propolis (Hocking and Matsumura, 1960; Hodges et al., 2001; Ali, 2012; Mortensen et al., 2013).

Colony management and life cycle of *Apis mellifera*

There are several hive designs used for *A. mellifera* management such as the Gravenhorst hive, Huber Leaf hive, and the Langstroth hive (Langstroth and Dadant, 1922). When *A. mellifera* colonies are new, they are typically kept in small hive boxes called nucs with 5 frames until their population increases enough for a beekeeper to move them into multiple 8-10 frame hive boxes (Winston, 1991a). When there are at least 10 frames of brood and the colony may become overcrowded and swarm as a means of colony reproduction (Fefferman and Starks, 2006; Skinner et al., 2017). *Apis mellifera* hives consist of frames covered in wax cells that are drawn out by worker bees as either pollen, honey, or bee brood cells (Winston, 1991a). The bees use the wax frame to store pollen, make honey, and house their developing generations.

The entire juvenile lifecycle of *A. mellifera* takes place within wax cells in the hive. A single *A. mellifera* queen is either introduced to the colony via a beekeeper or the worker bees create specialized cells called queen cells (Winston, 1991b). In the queen cell, the worker bees place a less than two-day old larvae in the cell and feed it a special diet called royal jelly (Winston, 1991c). Royal jelly consists of a mixture of 50-60% H_2O, 10% proteins, 15% carbohydrates, 36% lipids, 1.5% mineral salts, vitamins, and bioactive substances (Viuda-Martos et al., 2008). When the colony creates a queen, they usually create multiple queen cells

to ensure that at least one will survive (Mortensen et al., 2013). Once a new queen emerges after pupating for 8 days, she kills the remaining developing queens (Mortensen et al., 2013). The queen communicates and controls the entire colony with pheromones. The new queen then goes on two to three mating flights before beginning to lay eggs (Mortensen et al., 2013). She lays one egg per cell and worker bees then keep the eggs clean and feed the developing larvae brood food, honey, and pollen for the remainder of the larval stage (Mortensen et al., 2013). In the spring and summer, the queen will lay both fertilized and unfertilized eggs that take 3 days to hatch (Winston, 1991b). The fertilized eggs develop into female worker bees or queens and the unfertilized eggs develop into male drones (Winston, 1991b). The sole purpose of drones is to leave the colony and mate with a queen of another colony (Winston, 1991d). Most drones that survive into late fall will be evicted from the hive by the female worker bees. The female worker bees are capable of laying eggs if the colony has lost their queen; however, they will all develop into drones as the female worker bees are not capable of mating (Winston, 1991c). The female worker bees switch jobs depending on their age (Winston, 1991e). Worker bee jobs include cleaning wax cells, drawing out wax cells, feeding developing larvae, foraging, and defending the hive (Winston, 1991e).

The duration of larval development is determined by the type of the individual. Drone eggs hatch in three days, they develop as larva over seven days, cap their cell in two days, molt into an adult after ten days, and eclose two days later as a sexually mature adult male (Winston, 1991b). Worker bee eggs hatch in three days, larva develop over six days, cap their cell in one day, molt into an adult after 10 days, and eclose one day later as a mature adult female (Winston, 1991b). Queen eggs hatch in three days, larva develop over five days, cap their cell in one day, molt into an adult in six days and eclose in one day (Winston, 1991b). Healthy queens will live

for an average of 3-4 years, female worker bees will live for an average of 36 days in the summer and 6 months in the winter, and drones will live for an average of 22 days in the summer and 59 days if they survive through the winter (Winston, 1991b). *Apis mellifera* colonies can be bred for certain qualities such as increased honey production and resistance to stressors that may affect colony health (Brascamp and Bijma, 2019).

Factors affecting *Apis mellifera* colony health

Colony strength, in terms of number of working bees on a frame, is typically a good indicator of colony health (Smith et al., 2013). Colony health is dependent on the entire colony's abilities to survive abiotic and biotic stressors (Smith et al., 2013). Abiotic stressors typically include weather such as heavy rain, strong winds, tornados, accidental exposure to chemicals from crop overspray or contaminated water (Krupke and Long, 2015), as well as incorrect application of chemicals by the beekeeper (Mullin et al., 2010; Zhu et al., 2014). In North America, biotic stressors include the bacterias *Paenibacillus larvae* (Ash et al., 1993) and *Melissococcus pluton* (Trüper and de' Clari, 1998), the fungi *Ascosphaera apis* (Spiltoir and Olive, 1955), *Nosema ceranae* (Fries et al., 1996), and *Nosema apis* (Zander, 1909), mites such as *Acarapis woodi* (Rennie, 1921) and *Varroa destructor* (Anderson and Trueman, 2000), insect pests such as *A. tumida* (SHB) (Murray, 1867) and *Galleria mellonella* (Linnaeus, 1758), and mammalian pests such as mice, skunks, and bears (Sanford, 1987; Spivak and Reuter, 2016). Strong, healthy colonies experience the same pressure from these stressors as weaker colonies; however, strong colonies may survive these stressors due to population size (Smith et al., 2013).

In Alabama, all of the above biotic and abiotic stressors are present. Cultural, mechanical, chemical, and biological management for these stressors varies depending on pest type and importance. Chemical controls for *A. mellifera* pests are available; however, chemical residues

may affect colony health and hive product quality if used improperly. Fahey et al. (2019) released a survey conducted in 2017-2018 in 38 states, including Alabama, and found that 100% of wax sampled from where *A. mellifera* brood are raised contained active ingredients (a.i.) Fluvalinate, Coumaphos, Thymol, and Amitraz metabolite, 2,4 Diamethylphenyl formamide. These a.i. are common in miticides used to control *V. destructor*. In a survey of honey bee colonies in North America, one *A. mellifera* stock developed by the USDA (Rinderer et al., 2005) and one stock developed in Alabama (Calvert Apiaries, Calvert, AL) showed resistance towards one pest, *V. destructor*, because of a varroa sensitive hygiene gene that has developed through breeding research (Ward et al., 2008). Breeding *A. mellifera* colonies to maximize hygienic behavior is one step towards limiting the use of chemicals in and around colonies. Another important factor of managing pests of *A. mellifera* colonies is the ability for colonies to swarm. If a colony swarms, it is likely that their pests will follow. One study in Alabama found that *A. mellifera* colonies can occupy natural cavities or man-made boxes that are only 5-6.7 liters (Prange and Nelson, 2007). This is important because pests of *A. mellifera*, such as SHB, are capable of infesting and reproducing within wild colonies, making any eradication efforts impossible once they are established (Zawislak, 2014; Willcox et al., 2017).

Aethina tumida the small hive beetle

An important pest of *A. mellifera* colonies in Alabama is SHB, a beetle native to sub-Saharan Africa in the order Coleoptera (Linnaeus, 1758), and family Nitidulidae (Latrielle, 1802). SHB was first documented in the United States through species collected in Florida in 1998; however, previously unidentified beetle specimens collected in Charlston, South Carolina in 1996 and 1997 were later identified as SHB (Hood, 2004). Some literature suggests that SHB was officially listed as an invasive species in 1996, though the literature that suggests this does

not provide details as to where it is listed as such (Neumann et al., 2016; Shäfer et al., 2019). The current distribution of SHB also presents conflicting information. Some literature states that SHB currently inhabits both managed and wild bee colonies in every continent except Antarctica (Ellis and Ellis, 2010; Neumann et al., 2016; Shäfer et al., 2019) while others state that the distribution is only in parts of Africa, North America, and Australia (Hood, 2004; Neumann and Elzen, 2004; Ellis and Ellis, 2010; Ellis, 2012; Neumann et al., 2013; Willcox et al., 2017).

Life cycle of *Athena tumida*

SHB spreads via sexually mature adults emerging from the soil they pupated in, flying to a suitable hive, and ovipositing directly on frames within the hive. Mating occurs once sexually mature male and female SHB are within a suitable hive (Neumann and Elzen, 2004; Mustafa et al., 2015). The general lifecycle of SHB includes five stages: egg, larvae, wandering larvae, pupa, and adult (Fig. 1). Sexually mature females lay up to 2,000 eggs within their lifetime (Ellis, 2004). They will either oviposit directly onto frames if no worker bees are present or will oviposit directly onto the *A. mellifera* pupa by chewing a hole in either the top or side of a capped cell (Ellis, 2004; Ellis et al., 2004a). SHB eggs are ~1.4 mm long and ~0.26 mm wide and hatch anywhere from twenty-four hours to three days depending on temperature and humidity (Neumann et al., 2013, 2016). Larva grow from 1.3 mm to ~8.6 to 10.5 mm in length over three to thirty days depending on food availability and temperature; the mature wandering larva may survive up to sixty-one days without food (Neumann et al., 2013, 2016). A larva is considered a wandering larva once it is no longer eating and begins to travel away from the food source in search of a suitable pupation location. The pupation stage can take thirteen to seventy-four days depending on temperature and soil moisture (Neumann et al., 2013). SHB adults are 5

to 7 mm in length and 3 to 4.5 mm in width and can live one to twelve months (Neumann et al., 2013, 2016).

Infestation process of *Athena tumida*

SHB are opportunistic pests of wild and domesticated social bee colonies and the infection process is the same for any colony they invade. The infection process for SHB in a domesticated *A. mellifera* colony begins when an adult SHB flies to a suitable hive, likely found through their olfactory senses, and lays eggs within the colony (Graham et al., 2011). Both adult and larva SHB feed on honey, pollen, and bee brood and female adult SHB oviposit directly onto wax comb that may contain all three (Ellis et al., 2002; Neumann et al., 2013). Adults do not cause substantial damage to the hives, they only feed, mate, and lay eggs in the comb (Neumann et al., 2013). The larval stage is the most destructive stage of SHB as they consume the hive products which leads to bee brood death, honey fermentation from fecal material, unmarketable wax, and slimy comb (Fig. 2) (Neumann et al., 2013; Zawislak, 2014). SHB adults reproduce in the spring, summer, and fall and are known to overwinter inside *A. mellifera* hives (Neumann et al., 2016) Some sources believe that SHB pupa may overwinter in clusters within *A. mellifera* colonies (Pettis and Shimanuki, 2000; Shäfer et al., 2011; Atkinson and Ellis, 2012).

Control measures and regulations for *Athena tumida*

There is no known threshold for SHB damage as the economic damage caused by this pest is primarily determined by the health of the *A. mellifera* colony at the time of infection (Ellis et al., 2003; Zawislak, 2014).

Cultural control methods for SHB include management of *A. mellifera* colonies, reducing stress from disease or other pests, and capitalizing on the natural hygienic behavior of *A.*

mellifera. As opportunistic pests, adult SHB are only successful in infesting *A. mellifera* colonies if conditions within the hive are favorable for the SHB. These favorable conditions are created by improper hive management by the beekeeper, some other factor that has weakened the colony such as weather or a previous pest weakening the colony, or an *A. mellifera* colony with unhygienic behavior (Ellis, 2012; Cuthbertson et al., 2013; Zawislak, 2014). Improper management includes keeping colonies in the proper sized hive for the colony's size, monitoring for pests, and ensuring there is a queen present and laying eggs. If an *A. mellifera* colony is in the proper sized hive for their population size and demonstrates hygienic behavior, the *A. mellifera* workers will remove SHB larva from the hive (Ellis et al., 2004a). The worker bees will also find SHB adults in the hive and herd them into sections of the hive box where the workers will seal the SHB in a cell-like structure with propolis (Neumann and Elzen, 2004). Small *A. mellifera* colonies are unable to patrol the hive for pests like SHB and are more likely to be infested (Neumann et al., 2016). Some *A. mellifera* colonies demonstrate unhygienic behavior and do not remove SHB larva or trap adults, allowing the infestations to progress. Unhygienic behavior has been observed in both strong and weak colonies, and the behavior is generally attributed to the genetics of the bees themselves (Smith et al., 2013). The overall health of *A. mellifera* colonies determine how well the colony will handle a SHB infestation (Ellis et al., 2003). For example, a healthy *A. mellifera* colony may experience the same pressure from SHB adults and larva as an unhealthy colony but the healthy colony may survive with the infestation for the year while the weaker colony may die in a week. Strong populations of *A. mellifera* within a hive may be able to control SHB larva and adults better than weaker *A. mellifera* colonies because of the bees' sheer number and reproductive abilities (Ellis et al., 2003). If the SHB infestation is not resolved through *A. mellifera* hygienic behavior or is caused by a factor that cannot be resolved naturally, other control measures must be implemented.

Mechanical control includes moving damaged frames from a weak *A. mellifera* colony into a healthy colony if they can be salvaged, freezing the comb if the damage is too severe, to kill any SHB eggs, larva, and adults that may be on the comb before recycling the wax, or smashing adults as they are seen in and around the hive. Mechanical control also includes monitoring where the beekeeper notes how many SHB they removed from the hive during maintenance. Physical monitoring devices may also be placed inside the hive to entrap SHB adults. Some mechanical controls include the use of vinegar, diatomaceous earth, or mineral oil inside of the traps to help capture SHB adults while not harming the *A. mellifera* colony. Some traps are available to place under the hives as a tray to catch and trap SHB wandering larvae as they drop below the hive.

Chemical treatments inside and around *A. mellifera* hives are heavily regulated due to the insecticidal properties that may affect *A. mellifera* on an individual and whole-colony level. Hive products such as honey, wax, propolis, and the wood materials that the hive boxes are made of have also been known to collect chemical residues, creating high levels of concentrated a.i. over time (Berry et al., 2013; Fulton et al., 2019). In Alabama, chemical controls include the insecticide CheckMite+® (10% Coumaphos) (Bayer CropScience, Raleigh, NC) inside of the hive and the insecticide GaurdStar (40% Permethrin) (Y-TEX Corporation, Cody, Wyoming) around the outside of the hive and around beekeeping supply storage areas. Chemical treatments are no longer advised due to the toxicity of the chemicals to *A. mellifera*, the possibility that the chemicals taint hive products, and the ability for SHB to develop resistance (de Guzman et al., 2001; Cuthbertson et al., 2013).

The current distribution of SHB is heavily disputed. Countries that are not believed to currently have established SHB infestations have created regulations that attempt to prevent or

eradicate any introductions from movement of hive materials into their country (Mutinelli et al., 2017). These regulations include governmental monitoring of all *A. mellifera* colonies for the presence of SHB adults or larva in or around the hives (Mutinelli et al., 2014; Shäfer et al., 2019). If SHB are found, control methods range from burning entire colonies to freezing colony products, such as honey, before extracting (Mutinelli et al., 2017; Shäfer et al., 2019). Whether these eradication efforts are successful is difficult to determine due to the ability of adult SHB to fly and live in wild bee colonies (Zawislak, 2014; Willcox et al., 2017). SHB have been found in wax, comb, and adult *A. mellifera* shipments and eradication efforts are not feasible in most places once it has dispersed to natural bee colonies (Animal and Plant Health Inspection Services, 2004). This means that monitoring for and controlling SHB in colonies is the only option for the U.S. and other countries where SHB are widely established.

With no established threshold for SHB, beekeepers in Alabama are reliant on monitoring systems and the overall strength of their colonies to control this pest. Both commercial and hobby beekeepers have issues with SHB and with rising concern of factors that cause *A. mellifera* colony numbers to decline, any factor that has the ability to weaken a colony, or is an indicator that a colony is weak, should be examined. Adding a biological control agent to the list of current control measures might enhance the IPM program to control SHB in all life stages.

Entomopathogenic Nematodes

One potential biological control agent for SHB globally are entomopathogenic nematodes (EPNs). Nematodes that feed on invertebrates, such as insects, are globally distributed and are designated as entomopathogenic (Adams and Nguyen, 2002). All nematodes belong to the kingdom Animalia and phylum Nematoda (Rudolphi, 1808; Stock and Goodrich-Blair, 2012).

The taxonomic classification has been updated as new technologies have advanced our understanding of EPN relationships.

Currently, the family Steinernematidae comprises the two genera *Steinernema*, with approximately ninety-five described species, and *Neosteinernema*, with only one described species that specializes on termites (Adams and Nguyen, 2002; Stock and Goodrich-Blair, 2012; Hunt, 2016; Abd-Elgawad, 2019). The family Heterorhabditidae only encompasses the family *Heterorhabditis* (Poinar, 1975), which has sixteen species (Hunt, 2016). Hunt (2016) discusses all described EPN species within the three genera in an effort to provide a more concise view of species diversity. He found that the genus *Steinernema* appears to be far more speciose than *Heterorhabditis* and he speculated that this may be caused by slight differences in lifecycle and infection processes.

Infection process and lifecycle

EPNs have two different hunting mechanisms – some are cruiser (seek and attack) predators while others are ambush (sit and wait) predators (Shapiro-Ilan et al., 2002). *Steinernema* spp. are ambush predators whereas *Heterorhabditis* spp. are cruiser predators, though there are some species within *Steinernema* that demonstrate both hunting techniques (Table 1) (Lewis et al., 1992; Ellis et al., 2010; Wilson et al., 2012). *Steinernema* spp. host presence and frequency uncertainty may be one of the reasons they developed more species over time (Hunt, 2016). One aspect all EPN genera have in common is that they have a symbiotic bacterium living in their digestive tract that is responsible for killing the insect host as well as providing an environment for the EPNs to reproduce. The exact bacterial genus and species is, for the most part, dependent on the nematode genus and species. *Steinernema* spp. carry bacteria in the genus *Xenorhabdus* spp. (Thomas and Poinar, 1979) and *Heterorhabditis* spp. carry

bacteria in the genus *Photorhabdus* spp. (Table 1) (Boemare et al., 1993) (Akhurst, 1982; Kaya and Gaugler, 1993; Boemare et al., 1996; Adams and Nguyen, 2002; Stock, 2019). Both bacteria are gram-negative and belong to the family Enterobacteriaceae (Forst and Clarke, 2002). The bacteria lives within the nematode as two variants, phase one and phase two, with different physiological biochemical properties for each (Akhurst and Boemare, 1990; Chen et al., 1994; Forst and Clarke, 2002). The phase one variants of *Xenorhabdus* spp. and *Photorhabdus* spp. produce a toxin that kills the host and produce antibiotics that prevent other microorganisms such as yeast and a wide range of bacteria including *Bacillus* spp. (Cohn, 1872) from growing within the host cadaver (Akhurst, 1982; Akhurst and Boemare, 1990; Chen et al., 1994; Boemare, 2002; Forst and Clarke, 2002; Wesche et al., 2019). The phase one bacterium also affects dye absorption, pigmentation, and provides ideal environmental conditions for nematode reproduction that the phase two variants do not (Zhang et al., 2019). Most literature suggests that the phase two variants may be pathogenic to insect hosts; however, EPN species are generally associated with the phase one bacterial cells (Akhurst, 1982; Akhurst and Boemare, 1990; Chen et al., 1994; Wesche et al., 2019; Zhang et al., 2019). However, Blaxter et al. (1998) and Forst and Clarke (2002) claim that while *Heterorhabditis* spp. are unable to retain phase two bacteria, *Steinernema* spp. can. Forst and Clarke (2002) also distinguished between the physiological properties of *Xenorhabdus* spp. and *Photorhabdus* spp. One of the noted differences they found was the specificity of the symbiotic relationships between the EPN species and the bacteria species. The symbiotic relationship between *Photorhabdus* spp. and *Heterorhabditis* spp. is restrictive as only one species of nematode will only retain one species of bacteria (Forst and Clarke, 2002). Whereas, one species of *Steinernema* may develop on and retain the same *Xenorhabdus* spp. as another species of *Steinernema* (Forst and Clarke, 2002). In their infective juvenile (IJ) stage, *Steinernema* spp. carry their symbiotic bacterium in a specialized intestinal

vesicle while *Heterorhabditis* spp. carry their symbiotic bacterium in the last 2/3 of intestines (Forst and Clarke, 2002).

Infection of the insect occurs when the third-stage IJ (IJ3) EPN encased in its second stage cuticle finds prey via host released chemoattractants and enters through a natural orifice (Fig. 3) (Gaugler et al., 1989; Kaya and Gaugler, 1993; Boemare, 2002). The EPN releases the symbiotic bacteria into the insect hemocoel and the bacteria kill the insect through toxemia or septicemia within 48 hours and create suitable breeding conditions for the EPN (Fig. 3) (Gaugler et al., 1989; Kaya and Gaugler, 1993; Boemare, 2002). First generation EPNs feed and molt into fourth-stage juveniles before molting again into sexually mature adults (Smart, 1995). Generally, *Heterorhabditis* spp. first generation adults are hermaphroditic while first generation *Steinernema* spp. adult populations contain both males and females (Forst and Clarke, 2002; Hunt, 2016). Once the host cadaver is suitable for reproduction, eggs are laid inside the host (Smart, 1995). The eggs emerge as first-stage juveniles (IJ1) (Smart, 1995). They feed and molt into second-(IJ2), IJ3, and fourth-stage juveniles (IJ4) before molting into males or females of the second generation (Smart, 1995). The second-generation mates and the females lay eggs that hatch into the third generation of IJ1 (Smart, 1995). The IJ1 feed and molt into IJ2 that feed and take up a small amount of living bacteria into their digestive tract (Smart, 1995). These third generation IJ2 molt into IJ3, retaining their IJ2 cuticular sheath, and exit the cadaver in search of a new host (Smart, 1995). The bacteria rely on the EPN for protection and transportation into hosts and the EPNs rely on their symbiotic bacteria to kill the insect and create a suitable environment for reproduction of two to three generations within a single prey (Kaya and Gaugler, 1993). Screening EPNs for biological control of specific pest insects is important because some EPN species are generalists while others are specialists.

EPNs as biological control agents

Laboratory research in Europe and the U.S. have shown great promise for four species of *Steinernema* and two species of *Heterorhabditis* for controlling the wandering larva and pupation stage of SHB (Ellis et al., 2010; Cuthbertson et al., 2012; Shapiro-Ilan et al., 2014; Alonso et al., 2018). Both *Steinernema* spp. and *Heterorhabditis* spp. are currently marketed biological control agents for pest insects based on their individual host preference and are reared and marketed for control of agricultural pests (Adams and Nguyen, 2002; Alonso et al., 2018). Examples of products available for use in North America include NemaSeek™ (ARBIO Organics, Tucson, Arizona), which contains individual strains of *H. bacteriophora* (Poinar, 1975), *H. indica* (Poinar et al., 1992), or *S. kraussei* (Steiner, 1923) and NemAttack™ (ARBIO Organics, Tucson, Arizona), which contains individual strains of *S. carpocapsae* (Weiser, 1955), *S. feltiae* (Filipjev, 1934), or *S. riobrave* (Cabanillas et al., 1994). Of the above products, only NemaSeek™ with *H. indica* and NemAttack™ with *S. riobrave* are marketed for use on SHB. Another EPN product marketed for control of SHB is Grub-Away® Nematodes (Gardens Alive!® Inc. Lawrenceburg, Indiana), which contains *H. bacteriophora*. EPNs may also be purchased in products with mixed genera and species from companies such as ARBICO Organics, Buglocical Control Systems (Tucson, Arizona), and Bugs for Growers LLC (Strongsville, Ohio). The previously mentioned products sell EPNs in packages of five million to five hundred million individuals per package and the nematodes are shipped either suspended in a gel substance or a dry material that is to be mixed with water for application. Application rates vary drastically by company. For example, according to the labels, one package of five million *H. bacteriophora* in Grub-Away® Nematodes is said to cover 200 square feet while one package of five million *H. bacteriophora* in NemaSeek™ is said to cover 1,600 square feet. As each of the six EPN species mentioned above have different hunting styles, symbiotic bacteria species, host preferences, and environmental

preferences, it is important to understand how they perform as biological control agents individually before integrating them into an IPM program.

Heterorhabditis bacteriophora

Predation, limitations, and advantages

Heterorhabditis bacteriophora was first described in Australia (Adams and Nguyen, 2002) and is a cruise predator (Table 1) (Lewis, 2002; Tofangsazi et al., 2012). *Heterorhabditis bacteriophora* IJ3 are considered to have low desiccation, hypoxia, and UV tolerance, moderate heat tolerance, and moderate cold tolerance (Table 1) (Grewal, 2002). Laboratory bioassays determined that *H. bacteriophora* survives best at 7.5°C and least at 25°C (Strauch et al., 2000). One limitation of *H. bacteriophora* is that host cadaver desiccation may reduce their reproduction potential (Spence et al., 2010). In Poland, the effects of twenty-one agrochemicals on *H. bacteriophora* IJ3 survival and virulence was tested (Table 2) (Radová, 2011). Of the agrochemicals tested, products with the a.i. nuarimol (0.05 l/Kg/ha), diafenthiuron (0.08 l/Kg/ha), kinoprene (0.075 l/Kg/ha), methomyl (0.15 l/Kg/ha), tebufenozide (0.01 l/Kg/ha), and pyriproxyfen (0.025 l/Kg/ha) caused IJ3 mortality up to 17.92% (Radová 2011). Petrikovszki et al. (2019) determined that the high doses (>0.3%) of the natural pesticide *A. indica* extract (active compound azadirachtin) resulted in 97.5% mortality of *H. bacteriophora* (Table 2). Barbercheck et al. (1995) found that the diet of the insect host may have a positive or negative effect on *H. bacteriophora* reproductive capabilities. Duncan et al. (1996) found that *H. bacteriophora* prefer shaded soils but some literature suggests that *H. bacteriophora* can be bred for temperature and desiccation tolerance (Ehlers et al., 2005; Mukuka et al., 2010). Research on effects of acaricides on *H. bacteriophora* found that acaricides with the a.i. abamectin, pyrethrin, fenpyroximate, have no effect on mortality or efficacy (Table 1) (Laznik and Trdan, 2017). This

is an advantage for *H. bacteriophora* as it can be used along-side some acaricides in an IPM program.

Bacterial symbionts and use as biological control agent

Poinar et al. (1977) isolated a bacterium they called *X. luminescens,* which was later changed to *Photorhabdus luminescens* by Boemare et al. (1993) (Table 1). There are two subspecies of *P. luminescens* that are known symbionts with different subgroups of *H. bacteriophora. Photorhabdus luminescens* subspecies *luminescens* (Thomas and Poinar, 1979) is symbiotic with *H. bacteriophora* in the subgroup Brecon and *P. luminescens* subspecies *laumondii* (Fischer-Le Saux et al., 1999) is symbolic with *H. bacteriophora* in the subgroup HP88 (Koppenhöfer, 2007). *Heterorhabditis bacteriophora* in the subgroup HP88 is found in North and South America, Southern Europe, and Australia (Boemare, 2002). In Turkey, *H. bacteriophora* is associated with *P. luminescens* subsp. *thracensis* (Hazir et al., 2004) and *P. temperate* (Fischer-Le Saux et al., 1999) is the symbiont for *H. bacteriophora* subgroup NC (Koppenhöfer, 2007). In laboratory studies, *H. bacteriophora* CCA strain was an effective biological control against *D. abbreviates* (Duncan and McCoy, 1996; Shapiro-Ilan et al., 1999), *Boophilus microplus* (Lahille, 1905) (de Oliveira Vasconcelos et al., 2004), Coleoptera: Scarabaeidae (Latreille, 1802) larvae (Koppenhöfer and Fuzy, 2006), and *A. aegypti* (Chaudhary et al., 2017). In North America, *H. bacteriophora* is a promising biological control agent against *C. nenuphar* (Shapiro-Ilan et al., 2011). Askary and Abd-Elgawad (2017) listed ten agriculturally important insect hosts that could be controlled by *H. bacteriophora*. Of the ten, insects that belong to Coleoptera: Scarabaeidae, *Bradysia* spp., *D. abbreviates,* and *Sphenophorus* spp. (Schönherr, 1838) are found in Alabama. The NemaSeek™ (ARBIO Organics, Tucson, Arizona) label lists ~32 insect genus and species that can be controlled by *H. bacteriophora*, none of which were SHB. Grub-Away® Nematodes product label lists nine insects susceptible to *H.*

bacteriophora including SHB with an application rate of five million nematodes per 200 square feet (approximately nine *A. mellifera* hives). Laboratory bioassays in Australia; however, found that *H. bacteriophora* strain NJ, was not a viable biological control agent of SHB (Spooner-hart, 2008).

Heterorhabditis indica

Predation, limitations, and advantages

Heterorhabditis indica was first described in India (Adams and Nguyen, 2002) and is considered a cruiser predator (Table 1) (Raveendranath et al., 2007). They survive best in 15°C and desiccation occurs at 5°C (Strauch et al., 2000). *Heterorhabditis indica* IJ3 are considered to have moderate desiccation and hypoxia tolerance, low UV and heat tolerance, and high cold tolerance (Table 1) (Grewal, 2002). Laboratory studies in 2005 observed the effect of temperature and relative humidity on *H. indica* IJ3 emergence and found that they can only emerge in temperatures between 15-30°C and 85-100% relative humidity (Lalramliana et al., 2005). The effects on IJ3 virulence and mortality were also tested on fourteen insecticides, six fungicides, and seven herbicides used in rice systems (Table 2) (Chavan et al., 2018). The majority of insecticide and fungicide products tested caused 10% mortality or less after 72 hours. However, the insecticides with a.i. monocrotophos 36% and cartap hydrochloride 50% caused 19.5% and 100% mortality respectively and the fungicides containing the a.i. tricyclazole 75% and carbendazium 12% + mancozeb 3% caused 14.5% and 21.5% mortality respectively (Chavan et al., 2018). Of the herbicides, products with the a.i. pendimethalin 30% caused 18% mortality (Chavan et al., 2018). One advantage is that *H. indica* can obtain up to 100% efficacy when applied in combination with some fungal and bacterial agents (Sankar et al., 2009). The fungal agents are *Meterhizium anisopliae* (Sorokin, 1883), *Beauveria bassiana* (Balsamo-Crivelli,

1835), or *Trichoderma viride* (Persoon, 1794) all applied at the field recommendation rate 1 X 10^9 spores/ml, and the bacterial agent is *Pseudomonas fluorescens* (Migula, 1894) at the field recommendation rate of 1 X 10^9 colony-forming unit per ml. (Table 2) (Sankar et al., 2009).

Bacterial symbionts and use as biological control agent

Photorhabdus luminescens subspecies *akhurstii* (Fischer-Le Saux et al., 1999) is the symbolic bacterium of *H. indica* (Table 1) *(*Koppenhöfer, 2007). In North America, *H. indica* can be used to control economically important pests such as *Corythucha ciliate* (Say, 1832) and *Stethobaris nemesis* (Prena and O'Brien, 2011) (Shapiro-Ilan and Mizell III, 2012), *D. abbreviates* (Shapiro-Ilan et al., 1999; Lacey and Shapiro-Ilan, 2003), *Helicoverpa armigera* (Hübner, 1808) (Divya et al., 2010), *C. nenuphar* (Shapiro-Ilan et al., 2011), and *Spodoptera litura* (Fabricius, 1775) (Divya et al., 2010; Askary and Abd-Elgaward, 2017). The NemaSeek™ label lists ten insects that are susceptible to *H. indica*, including SHB with an application rate of five million for every 218 square feet (approximately ten *A. mellifera* hives). *Heterorhabditis indica* has also shown promise as a suitable biological control agent for SHB in North America in both laboratory and field bioassays (Ellis et al., 2010; Shapiro-Ilan et al., 2010; Hill et al., 2016).

Steinernema carpocapsae

Predation, limitations, and advantages

Steinernema carpocapsae have undergone more than five name changes since their discovery (Bedding, 2006). Most literature suggests that *S. carpocapsae* is an ambush predator that prefers to hunt in upper soil levels (Table 1) (Lewis, 2002; Tofangsazi et al., 2012). However, Wilson et al. (2012) suggests that *S. carpocapsae* may also use cruising predator techniques in environments that lack mineral soils (Table 1). *Steinernema carpocapsae* IJ3 are

considered to have high desiccation, hypoxia, and UV tolerance, moderate heat tolerance, and high cold tolerance (Table 1) (Grewal, 2002). Jagdale and Grewal (2007) determined that desiccation of IJ3 begins below 5°C and above 35°C; however, virulence after desiccation only decreases if *S. carpocapsae* experiences temperatures above 35°C followed by temperatures below 5°C. Guy et al. (2009) states that *S. carpocapsae* tends to have low virulence around 15°C and high virulence around 20°C. They also found that virulence is greatly dependent on laboratory storage conditions and their findings are similar to Jagdale and Grewal (2007). Zhang et al. (1994) tested the toxicity of fourteen organophosphates, seven carbamates, four synthetic pyrethroids, and the a.i. imidacloprid and cartap on *S. carpocapsae* IJ3's (Table 2). Of the organophosphates, they found that diazinon (100μg/ml), dichlorvos (10 μg/ml., 100 μg/ml), fenthion (10 μg/ml., 100 μg/ml), malathion (10 μg/ml., 100 μg/ml), temephos (10 μg/ml), trichlorfon (10 μg/ml., 100 μg/ml), profenofos (10 μg/ml., 100 μg/ml), propetamphos (100 μg/ml), prothiofos (10 μg/ml., 100 μg/ml), and pyraclofos (10 μg/ml., 100 μg/ml) were toxic to *S. carpocapsae*. Of the carbamates, synthetic pyrethroids, and a.i. tested, only cartap (10 μg/ml., 100 μg/ml) was toxic. Gordon et al. (1996) found that the carbamates carbofuran and fenoxycarb cause mortality of IJ3's of *S. carpocapsae* All strain with the LD_{50} at 0.03mg/ml (Table 2). Barbercheck et al. (1995) found that the diet of the insect host may have a positive or negative effect on *S. carpocapsae* reproductive capabilities.

Bacterial symbionts and use as biological control agent

The bacterial symbiont for *S. carpocapsae* is said to be exclusively *X. nematophila* (Poinar and Thomas, 1965) (Table 1) (Chen et al., 1994; Boemare, 2002; Martens et al., 2004). Snyder et al. (2007) found that the infection of an insect host occurs when *S. carpocapsae* IJ3s defecate inside the insects hemocoel. Strains of *S. carpocapsae* were found to be virulent against

Synanthedon exitiosa (Say, 1824) in both laboratory and field trials (Cottrell and Shapiro-Ilan, 2006). Lacey and Chauvin (1999) determined *S. carpocapsae* is a viable biological control agent for *C. pomonella* in fruit bins. In the United Kingdom, *S. carpocapsae* is also used to control *H. abietis* (Torr et al., 2007). In North America, *S. carpocapsae* is a promising biological control agent against *C. nenuphar* (Shapiro-Ilan et al., 2011). Askary and Abd-Elgawad (2017) listed twenty-four suitable hosts of agricultural importance that could be controlled with *S. carpocapsae.* Of the twenty-four listed, *Agrotis ipsilon* (Hufnagel, 1766), *Plutella xylostella* (Linnaeus, 1758), *G. mellonella*, *Centrococcus* sp. (Borkhsenius, 1948), *Amyelois transitella* (Walker, 1863), *Platyptilia carduidactyla* (Riley, 1869), *Liriomyza* spp., *Callosobruchus* sp. (Pic, 1902), *C. formicarius, Scatella* spp., *Ctenocephalides felis* (Bouche, 1835), *C. pomonella, C. sordidus, H. zea, Holotrichia consanguinea* (Blanchard, 1850), *Chrysoteuchia topiaria* (Zeller, 1866), and *Scapteriscus* spp. are found in Alabama. The NemAttack™ label lists ~34 insect genus and species that can be controlled by *S. carpocapsae*, none of which were SHB. However, laboratory bioassay in Europe (Cabanillas and Elzen, 2006; Cuthbertson et al., 2012) and North America (Shapiro-Ilan et al., 2010) determined that *S. carpocapsae* is a suitable biological control agent for SHB.

Steinernema feltiae

Predation, limitations, and advantages

Steinernema feltiae (formerly *Neoaplectana carpocapsae* (Weiser, 1955)) are considered an intermediate predator, meaning they use both cruising and ambushing techniques to hunt for hosts (Table 1) (Lewis, 2002). *Steinernema feltiae* IJ3 are considered to tolerate moderate desiccation, hypoxia, and Ultra violet (UV), low heat tolerance, and high cold tolerance (Table 1) (Grewal, 2002). When a suitable host is found and invaded, other *S. feltiae* within range of attracting chemical signals will congregate within the host (Hussaini, 2017). Little is known

about the attracting signals and at what point of EPN population the signal switches to deter any new *S. feltiae* from the cadaver. Gaugler et al. (1989) determined that the host-finding skill of *S. feltiae* could be optimized through selective breeding. *Steinernema feltiae* reproduce at temperatures of 10-25°C, develop into adults at temperatures of 12-30°C, lose infectivity below 12°C and above 30°C, and desiccate at temperatures above 37°C (Hussaini, 2017). They are most effective in shaded ground from either foliage cover or mulch and they can be dispersed naturally via ants carrying infected cadavers to their nest or on the interior and posterior of earthworms (Shapiro-Ilan et al., 1995; Hussaini, 2017). Hussaini (2017) tested the effects of insecticides, acaricides, and fungicides on *S. feltiae* (Table 2). He found that *S. feltiae* are tolerant to insecticides with kinoprene, lufenuron, methomyl, metoxyfenozide, oxamyl, piperonyl-butoxide, pyriproxyfen, and tebufenpyrad as their a.i. They are also tolerant to acaricides with a.i. azocyclotin, clofentezin, diafenthiuron, etoxazole, fenbutatinoxide, fenpyroximate, and tebufenpyrad, as well as fungicides with a.i. captan, fenhexamid, kresoxim-methyl, and nuarimol (Hussaini, 2017). This means that *S. feltiae* could be used alongside some chemical treatments in an IPM program. One disadvantage noted by Gordon et al. (1996) is that the carbamates carbofuran and fenoxycarb cause mortality of *S. feltiae* Umea strain IJ3 with the LD_{50} at 0.03mg/ml (Table 2). *Steinernema feltiae* are also susceptible to poultry manure, *Stratiolaelaps miles* (Berlese, 1882a), insecticides containing the a.i. fenamiphos, and *Azadirachta indica* (Jussieu, 1832) (Table 2) (Hussaini, 2017). *Steinernema feltiae* virulence may be negatively affected by the presence of *Brassica carinata* (Braun, 1941), either used as a cover crop and tilled or applied as seed meal, due to high levels of glucosinolates (Henderson et al., 2009). Another disadvantage of *S. feltiae* are the IJ3 sensitivity to environmental changes such as presence or absence of water and solar radiation (Gaugler et al., 1989). IJ3's tend to stay in the

areas where they were inoculated regardless of host presence, therefore, any environmental changes to the inoculation area will affect virulence (Gaugler et al., 1989).

Bacterial symbionts and use as biological control agent

The bacterial symbionts of *S. feltiae* include isolates of *Xenorhabdus nematophilus* (Poinar and Thomas, 1965) and *X. bovienii* (Akhurst, 1983) (Table 1) (Poinar and Thomas, 1965; Buecher and Popiel, 1989; Boemare, 2002). *Steinernema feltiae* is considered a viable biological control agent because of its broad host range, ability to be mass produced, and high virulence (Gaugler et al., 1989). In 1986, a Mexican strain of *S. feltiae* was found to be a successful biological control agent for *Ceratitis capitate* (Wiedemann, 1824), *Dacus cucurbitae* (Coquillett, 1899), and *Dacus dorsalis* (Hendel, 1912) in Hawaii (Lindegren and Vail, 1986) as well as *G. mellonella* in Mexico (Dunphy and Webster, 1986). *Steinernema feltiae* was also deemed a successful biological control agent for *Megaselia halterata* (Wood, 1910) (Scheepmaker et al., 1998). Of thirty-eight beneficial and pest insect species screened by de Doucet et al. (1999), *S. feltiae* was successful in parasitizing thirty. Toepfer et al. (2005) found that *S. feltiae* was effective against *Diabrotica virgifera* (LeConte, 1868) in Europe. Askary and Abd-Elgawad (2017) listed eight agriculturally important insect hosts that could be controlled by *S. feltiae*. Of the eight, *Cylas formicarius* (Fabricius, 1798), *Scatella* spp. (Robineau-Desvoidy, 1830), *Liriomyza* spp. (Mik, 1894), *Bradysia* spp. (Winnertz, 1867), *Cydia pomonella* (Linnaeus, 1758), *Cosmopolites sordidus* (Germar, 1824), and *Helicoverpa zea* (Boddie, 1850) are found in Alabama. The NemAttack™ (ARBIO Organics, Tucson, Arizona) label lists ~19 insects that are susceptible to *S. feltiae,* none of which are SHB. Laboratory bioassays in Australia found that *S. feltiae* strain T319, was not a viable biological control agent of SHB (Spooner-hart, 2008). However, *S. feltiae* has shown promise as a suitable biological control agent for SHB in laboratory bioassay in Europe (Cuthbertson et al., 2012).

Steinernema kraussei

Predation, limitations, and advantages

Steinernema kraussei was the first EPN documented. It was first discovered in 1917 and again in 1927 by Travassos but was not described or named until 1923 by Steiner (Hunt, 2016). Steiner named the EPN *Aplectana kraussei* and in 1927, Travassos changed the genus to *Sterinernema* (Adams and Nguyen, 2002). *Steinernema kraussei* are considered a cruise predator that prefer coniferous forest areas (Torr et al., 2007) and have low desiccation tolerance (Table 1) (Nimkingrat et al., 2013). They are a cold-hardy species that can survive temperatures below 2.7°C (Edmondson et al., 2002) though virulence is decreased in temperatures between 15 and 20°C, suggesting they are not viable biological control agents in warm conditions (Guy et al., 2009). Laboratory and field bioassay determined that *S. kraussei* host-finding capabilities are limited by soil pH that is below 4.0 (Fischer and Führer, 1990). Petrikovszki et al. (2019) determined that the high doses (>0.3%) of the natural pesticide *A. indica* extract (active compound azadirachtin) can cause 46.5-100% mortality of *S. kraussei* (Table 2).

Bacterial symbionts and use as biological control agent

The bacterial symbiont of *S. kraussei* is *X. bovienii* (Table 1) (Akhurst, 1983; Boemare, 2002). In 1999, *S. kraussei* was determined a viable biological control agent for *C. pomonella* in fruit bins (Lacey and Chauvin, 1999). Due to the cold temperature tolerance of the species, *S. kraussei* has been used as a biocontrol agent against overwintering *Otiorhynchus sulcatus* (Fabricius, 1775) (Edmondson et al., 2002). In the United Kingdom, *S. kraussei* is used to control *Hylobius abietis* (Linnaeus, 1758) (Torr et al., 2007). In North America, *S. kraussei* is a promising biological control agent against *C. nenuphar* (Shapiro-Ilan et al., 2011). *Steinernema*

kraussei applicated in combination with *H. bacteriophora* are highly effective against *Aedes aegypti* (Linnaeus, 1762) (Chaudhary et al., 2017). The NemaSeek™ label states that *S. kraussei* can be used to control vine weevils, sawfly weevils, and *Cydia latiferreana* (Walsingham, 1879) but does not list SHB as a host. However, *S. kraussei* has shown promise as a suitable biological control agent for SHB in laboratory bioassay in Europe (Cuthbertson et al., 2012).

Steinernema riobrave

Predation, limitations, and advantages

Steinernema riobrave is considered an intermediate predator, meaning they use both cruising and ambushing techniques to hunt for hosts (Table 1) (Lewis, 2002). *Steinernema riobrave* IJ3 are considered to have moderate desiccation, hypoxia, and UV tolerance, high heat tolerance, and low cold tolerance (Table 1) (Grewal, 2002). *Steinernema riobrave* is also known to congregate within a host cadaver and reproduce in temperatures between 28-36°C (Shapiro-Ilan et al., 2002). Jagdale and Grewal (2007) determined that desiccation occurs below 5°C and above 35°C; however, virulence after desiccation only decreases if *S. riobrave* IJ3 experiences temperatures above 35°C followed by temperatures below 5°C. One disadvantage is *S. riobrave* virulence is negatively affected by the presence of *B. carinata,* either used as a cover crop and tilled or applied as seed meal, due to high levels of glucosinolates (Henderson et al., 2009). One advantage of *S. riobrave,* is their ability to tolerant exposure to UV light at 340 nanometers for up to three hours (Jagdale and Grewal, 2007). This means that *S. riobrave* should still be infective if applicated during daylight hours. Another advantage is that *S. riobrave* can complete its entire lifecycle in hosts less than 5 millimeters long, whereas other *Steinernema* spp. are incapable of producing offspring in hosts that small (Bastidas et al., 2014).

Bacterial symbionts and use as biological control agent

The bacterial symbiont of *S. riobrave* include isolates of *X. cabanillasii* (Tailliez et al., 2006) (Table 1) (Koppenhöfer, 2007). *Helicoverpa zea* was the first insect screened as a host for *S. riobrave* in Texas (Cabanillas et al., 1994). In Arizona and Texas, *S. riobrave* was found to be a successful biological control agent in both laboratory and field tests for control of *Pectinophora gossypiella* (Saunders, 1843) (Gouge et al., 1996). Shapiro et al. (2002) summarized the known hosts at that point to be *Diaprepes abbreviates* (Linnaeus, 1758) (Duncan and McCoy, 1996; Shapiro-Ilan et al., 1999; Duncan et al., 2003; Stuart et al., 2004; Kaspi et al., 2010), *Anthonomus grandis* (Boheman, 1843) (Cabanillas, 2003), and *Conotrachelus nenuphar* (Harris, 1841) (Shapiro-Ilan et al., 2004; Shapiro-Ilan et al., 2011). IJ3's also showed great virulence towards stored product pests such as *Plodia interpunctella* (Hübner, 1813) and *Tribolium castaneum* (Herbst, 1797) (Ramos-Rodríguez et al., 2007). Yu et al. (2010) found that three Subterranean termite species; *Heterotermes aureus* (Snyder, 1920), *Reticulitermes flavipes* (Kollar, 1837), and *Coptotermes formosanus* (Shiraki, 1909) are all susceptible to *S. riobrave* strains. Due to their ability to reproduce in hosts smaller than 5 mm in length, *S. riobrave* are effective against *Frankliniella schultzei* (Trybom, 1910) larvae (Bastidas et al., 2014). Askary and Abd-Elgawad (2017) listed four agriculturally important insect hosts that could be controlled by *S. riobrave.* Of the four, *C. nenuphar*, *H. zea*, and *Scapteriscus* spp. (Scudder, 1868) are found in Alabama. The NemAttack™ (ARBIO Organics, Tucson, Arizona) label lists ~23 insects that are susceptible to *S. riobrave* including SHB with an application rate of five million per 1,600 square feet (approximately seventy-three *A. mellifera* hives). *Steinernema riobrave* has also shown promise as a suitable biological control agent for SHB in laboratory bioassay in Europe (Cuthbertson et al., 2012), and in field and laboratory bioassays in North America (Ellis et al., 2010).

All six of the EPNs above are generally reared under laboratory conditions on *G. mellonella* larvae, which are also a secondary opportunistic pest of *A. mellifera* colonies like SHB. Rearing techniques for all of the EPNs are outlined in Freidman (1990), Lewis (2002), and Shapiro et al. (2011). The majority of previous research mentioned was conducted under sterile laboratory conditions; however, both laboratory screening and field studies that observed the effect of soil types concluded that soil conditions have an effect on EPN IJ3 viability and mortality (Duncan and McCoy, 1996; Lewis, 2002; Lacey and Shapiro-Ilan, 2003; Hill et al., 2016; Shapiro-Ilan et al., 2011). All six of the EPNs previously discussed have shown promise as possible biological control agents for SHB larva in autoclaved soil (Cabanillas and Elzen, 2006; Ellis et al., 2010; Shapiro-Ilan et al., 2010; Cuthbertson et al., 2012; Hill et al., 2016); however, there is limited knowledge on how different soil types and conditions may affect their efficacy. The question of how SHB larva and pupa as well as the individual EPNs interact within natural soil is necessary to better understand the practicality of using them as a biological control agent in an IPM system.

Soil Type

Efficacy of biological control agents, such as EPNs on SHB, depend on a variety of factors including host specificity, pest presence, and characteristics of the environment where the host and pest interact. Soil is made up of particles, water, organic matter, gases, and microorganisms (Barbercheck, 1992; Delgado and Gómez., 2016). All of these components put together in different combinations become the soil types that soil dwelling organisms such as SHB wandering larva and pupa, as well as EPNs, must move through at some point in their lifecycle. As the majority of the SHB lifecycle occurs within soil and the infection process of all

EPN species begins and ends in soil, it is important to understand how biotic and abiotic characteristics of soil affect each of these organisms.

Biotic factors

The biotic factors that affect EPNs within the soil include competition and natural enemies. The microorganism that make up soil may contain bacteria and fungi that feed on insects or nematodes (Viaene et al., 2006). Epsky et al (1988) found that *Alycus roseus* (Koch, 1842) and *Hypogastrura scotti* (Yosii, 1962) can complete their entire lifecycle on *S. feltiae* IJ3. Kaya (2002) summarizes other entomophageous biotic factors including protozoa *Pleistophora schubergi* (Zwölfer, 1927) and *Nosema mesnili* (Weiser, 1961) and fungi *Arthrobotrys oliogospora* (Fresenius, 1850), *A. dactyloidesm, Monacrosporium ellipsosporum* (Grove, 1886), *M. cionopagum* (Drechsler, 1950), and *Drechmeria* spp. (Gam and Jansson, 1985). Other biotic factors include mites *Gamasellodes vermivorax* (Walter, 1987) and *Eugamasus* sp. (Berlese, 1892b), collembolan *Folsomia candida* (Willem, 1902) and *Sinella caeca* (Schött, 1896), tartegrades *Macrobiotus richtersi* (Murray, 1911), and other nematodes belonging to *Clarkus* sp. (Jairajpuri, 1970) and *Actinolaimus* sp. (Cobb, 1913).

Not all biotic factors have a negative effect on EPNs. For example, even though entomophageous mites such as *A. roseus* consume *S. feltiae,* IJ3 of the species have been observed attached to, and traveling on, the dorsum of *A. roseus*, consequently aiding in their dispersal (Epsky et al., 1988). Shapiro-Ilan et al. (1995) found that *S. carpocapsae* and *S. feltiae* horizontal dispersal was also aided by *Lumbricus terrestris* (Linnaeus, 1758). Various species of ants have also been known to carry EPN infected insect cadavers (Kaya, 2002; Hussaini, 2017).

Abiotic factors

Soil composition is important when discussing EPN efficacy because it is a factor that affects nematode mobility, infectivity, and mortality. Soils are comprised of many different particles that are various sizes. Typically, soil particle size (also called soil texture) is broken down into percent sand, percent silt, and percent clay which add up to 100% (Delgado and Gómez, 2016). According to Barbercheck (1992), sand particles range from 50 µm to 2000 µm, silt ranges from 2 µm to 50 µm, and clay is less than 2 µm in diameter. Kung et al. (1990a) observed the effects of autoclaved sand (92% sand, 4% silt, 4% clay), sandy loam (58% sand, 28% silt, 14% clay), clay loam (46% sand, 32% silt, 22% clay), and clay (30% sand, 44% silt, 26% clay) and found that *S. carpocapsae* survives best in sandy loam soils. Duncan et al. (1996) observed the efficacy of *S. riobrave* and *H. bacteriophora* in citrus groves containing Astatula fine sand (92% sand, 2% silt, 6% clay) and found both to be effective biological control agents. EPNs may have better mobility in sandy soils as they typically have larger pore space between each particle compared to silt and clay (Barbercheck, 1992). Divya and Sankar (2009) found that soil textures that are easier for nematodes such as EPN IJ3 to travel in, tend to aid in the IJ3 longevity. Soil throughout the entire state of Alabama has been grouped into fifteen types of soil with sand content ranging from 80% to 5%, silt content ranging from 71% to 10%, and clay content ranging from 53% to 7% (United States Department of Agriculture Natural Resource Conservation Service, https://www.nrcs.usda.gov/wps/portal/nrcs/surveylist/soils/survey/state/?stateId=AL).

For all soil textures, depth of soil and compaction of soil decrease the pore space (Barbercheck, 1992). This is important to know as different nematode species are adapted to hunt at different depths. Georgis and Poinar (1983) determined that *S. feltiae* infectivity occurs

between the first 20mm and 240mm of sandy soil and that newly emerged IJ3 tend to move up towards the surface of the soil once emerged. Laboratory studies on *H. bacteriophora* determined that they prefer hunting in soils heavy in clay (>15%) and silt (>80%) and above 100mm deep (Georgis and Gaugler, 1991; Shapiro-Ilan et al., 2000; Koppenhöfer and Fuzy, 2006). Field trials conducted in Florida by Duncan and McCoy (1996) found that *S. riobrave* remain in the top 30-150 mm of soil while *H. bacteriophora* prefer soil depth below 150 mm. Kaspi et al. (2010) confirmed that *S. riobrave* prefer soils with heavy sand, silt, and organic material and are most effective at a depth of 100mm of soil with efficacy decreasing as soil depth decreases. *Steinernema carpocapsae* prefers to hunt close to, and directly on, the soil surface (Moyle and Kaya, 1981; Divya and Sankar, 2009). However, Koppenhöfer et al. (1995) found conflicting data during their laboratory bioassay that supported *S. carpocapsae* infectivity increasing as soil depth increases. This conflict may have occurred as Koppenhöfer et al. (1995) were observing the effects of moisture levels within sandy loam, which is a factor Moyle and Kaya (1981) did not take into consideration.

Available moisture is another abiotic factor of soils that is important as EPNs need water to facilitate movement and survive. Water within soil is found between soil particles, which is where EPN propulsion occurs (Wallace, 1958; Koppenhöfer et al., 1995). Soils that only contain a thin layer of water between particles will restrict EPN movement and may cause them to enter an anhydrobiotic state (Koppenhöfer et al., 1995; Jagdale and Grewal, 2007; Wright and Perry, 2006). Gouge et al. (2000) determined that *S. riobrave* will move in order to remain in soils with a water potential range of 5.2% and 9.5% moisture. Molyneux and Bedding (1984) found that both *Heterorhabditis* spp. and *Steinernema* spp. parasitize better in loamy sand soils than those high in clay content. They speculated that moisture potential of different soil textures is the main factor affecting EPNs infectivity in different soil types. Kung et al. (1991) found that *S.*

carpocapsae has high infectivity at low soil moisture levels of 2%. Products used to improve soil drainage such as gypsum have a negative effect on mobility for EPNs that hunt near the surface as they create an environment with less water between soil particles (Kaspi et al., 2010). One aspect that may aid in increasing water retention in soil, consequently aiding EPN mobility, is organic material concentration (Koppenhöfer and Fuzy, 2006; Kaspi et al., 2010). Tofangsazi et al. (2012) state that the area where EPNs are applied in the field should be kept wet for at least 8 hours after application. Grant and Villani (2003) found that virulence of *H. bacteriophora*, *S. feltiae*, and *S. carpocapsae* in low moisture can be restored if the soil is rehydrated. One way to maintain moisture in soil after application is to apply EPNs in the early morning or evening when soil is less likely to dry out due to evaporation.

Temperature is another abiotic factor that affects EPN survival and infectivity. In a laboratory study, Molyneux (1985) found that *S. feltiae,* and two *Heterorhabditis* spp. were unable to infect an insect host in sand kept at 7% moisture at temperatures above 15°C. Burman and Pye (1980) speculate the main reasons for EPN decrease in infectivity in all soils was due to their inability to move, consumption of food reserves, and increased respiration rates. Kung et al. (1991) found that *S. carpocapsae* prefer soils between 5-25°C. Shapiro-Ilan et al. (2011) determined that results from laboratory trials on EPN control of *C. nenuphar* did not align with field trial results. In the follow up laboratory study they found that temperature had the greatest effect on the EPN species screened (Shapiro-Ilan et al., 2011).

Other abiotic factors that may affect EPN efficacy are soil salinity, organic material, pH, and UV exposure during application. Kaspi et al. 2010 found that *S. riobrave* are not able to infect a host in soils with high salt levels; however, when soil was washed, the EPNs regained their infective potential. Field studies by Bednarek and Gaugler (1997) determined that organic manure as a fertilizer increased the native population of *S. feltiae,* while fertilizers containing

nitrogen, phosphorus, and potassium suppressed *S. feltiae* and *H. bacteriophora.* Tofangsazi et al. (2012) outlined that EPNs have high infectivity in soils with a pH between 4 and 8 (Kung et al., 1990b) and they should be applied in the early morning or evening to avoid excessive UV exposure.

Abiotic soil factors that affect SHB pupation include soil texture, moisture, and temperature (Neumann et al., 2013; Neumann et al., 2016). SHB wandering larvae were thought to prefer to pupate in the first 100mm of light, sandy soil (Pettis and Shimanuki, 2000). However, Ellis et al. (2004b) observed the effects of four soil types, two moisture extremes (wet and dry), and two densities (packed and tilled). They found that SHB were only affected by soil moisture and that they were unable to pupate in dry packed and dry tilled soils. They also found that female SHB pupa spend less time in the soil than males. Frake and Tubbs (2009) observed SHB infestations in two apiaries with two different soil textures. They found there were greater amounts of SHB adults in an apiary located on silty clay and silty clay loam than in an apiary located on sandy loam and loam. They also found that across all four soil types, the SHB wandering larvae and pupa appeared in greater masses within the first 100 mm of soil, though some were also observed between 110 and 200 mm deep (Frake and Tubbs, 2009). Frake and Tubbs (2009) speculate that the SHB discrepancy they observed in adult beetles may have been influenced by the amount of moisture available in each of the soils. SHB pupation is directly influenced by soil temperature with pupation taking anywhere from 15 days during warm periods (Neumann et al., 2001) to 100 days during cold periods (Neumann et al., 2013).

Soil sterilization is necessary to create rearing standards for SHB and EPNs and to determine how individual soil factors effect an organism or system. However, non-sterilized soils

contain a multitude of biotic and abiotic factors that can greatly affect these organisms individually and together. Autoclaving is one effective method of sterilization that involves using a combination of heat and pressure to eliminate soil microorganisms within the soil (Wolf et al., 1989; Razavi and Lakzian, 2007). Autoclaving is most effective if the cycle is run two to three times (Wolf et al., 1989). Consequently, using an autoclave to sterilize soil alters the physical and chemical properties of the soil such as decreasing the pH, and increasing electrical conductivity, optical density, and extractible carbon and nitrogen (wolf et al., 1989; Razavi and Lakzian, 2007). Discrepancies have been observed between efficacy of EPNs in laboratory studies using autoclaved soil and efficacy in field trials. Understanding how natural, non-autoclaved soils effect both pest and biological control agent is important to determine if EPNs will be able to effectively control SHB larva and pupa. In Alabama, understanding effects of individual changes, such as moisture level, within different soil types on EPN efficacy is one step that will help to determine if EPNs are an effective addition to an IPM program to suppress SHB.

Research Objectives

The main objective in this study is to determine the efficacy of entomopathogenic nematodes controlling SHB larva in different soil types under low moisture conditions in order to improve current IPM practices. In order to achieve this, we will 1) determine the pupation success of SHB wandering larvae in autoclaved and non-autoclaved soil; 2) determine the efficacy of EPNs on SHB wandering larvae in autoclaved and non-autoclaved soil in low moisture conditions; and 3) determine the efficacy of EPNs on SHB wandering larvae in three non-autoclaved soil types at low moisture levels.

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Tables

Table 1: Bacterial symbionts, hunting style, tolerance levels, and habitat preferences of six entomopathogenic nematode species based on available literature (Lewis et al., 1992; Boemare et al., 1993; Duncan et al., 1996; Fischer-Le Saux, 1999; Strauch et al., 2000 Edmondson et al., 2002; Lewis, 2002; Grewal, 2002; Shapiro-Ilan et al., 2002; Ehlers et al., 2005; Lalramliana et al., 2005; Bedding, 2006; Jugdale and Grewal, 2007; Koppenhöfer, 2007; Raveendranath et al., 2007; Torr et al., 2007; Guy et al., 2009; Mukuka et al., 2010; Spence et al., 2010; Tofangsazi et al., 2012; Wilson et al., 2012; Nimkingrat et al., 2013; Adams, 2019)

			Tolerance					**Preferred habitat**
EPN species	**Bacterial symbiont**	**Hunting Style**	**Desiccation**	**Hypoxia**	**UV light**	**Heat**	**Cold**	**Soil**
H. bacteriophora	*P. luminescens*	Cruise	Low	Low	Low	Moderate	Moderate	Fine sand, heavy clay
H. indica	*P. luminescens*	Cruise	Moderate	Moderate	Low	Low	High	-
S. carpocapsae	*X. nematophila*	Cruise, ambush	High	High	High	Moderate	High	Sandy loam
S. feltiae	*X. nematophilus, X. bovienii*	Cruise, ambush	Moderate	Moderate	Moderate	Low	High	Shaded ground
S. kraussei	*X. bovienii*	Cruise	Low	-	-	Low	High	Coniferous forests
S. riobrave	*X. cabanillasii*	Cruise, ambush	Moderate	Moderate	Moderate	High	Low	Fine sand, silt

Table 2: Active ingredients of insecticides, acaricides, fungicides, and herbicides known to affect efficacy and virulence of six entomopathogenic nematode species (Zhang et al., 1994; Gordon et al., 1996; Sankar et al., 2009; Radová, 2011; Hussaini, 2017; Petrikovszki et al, 2019).

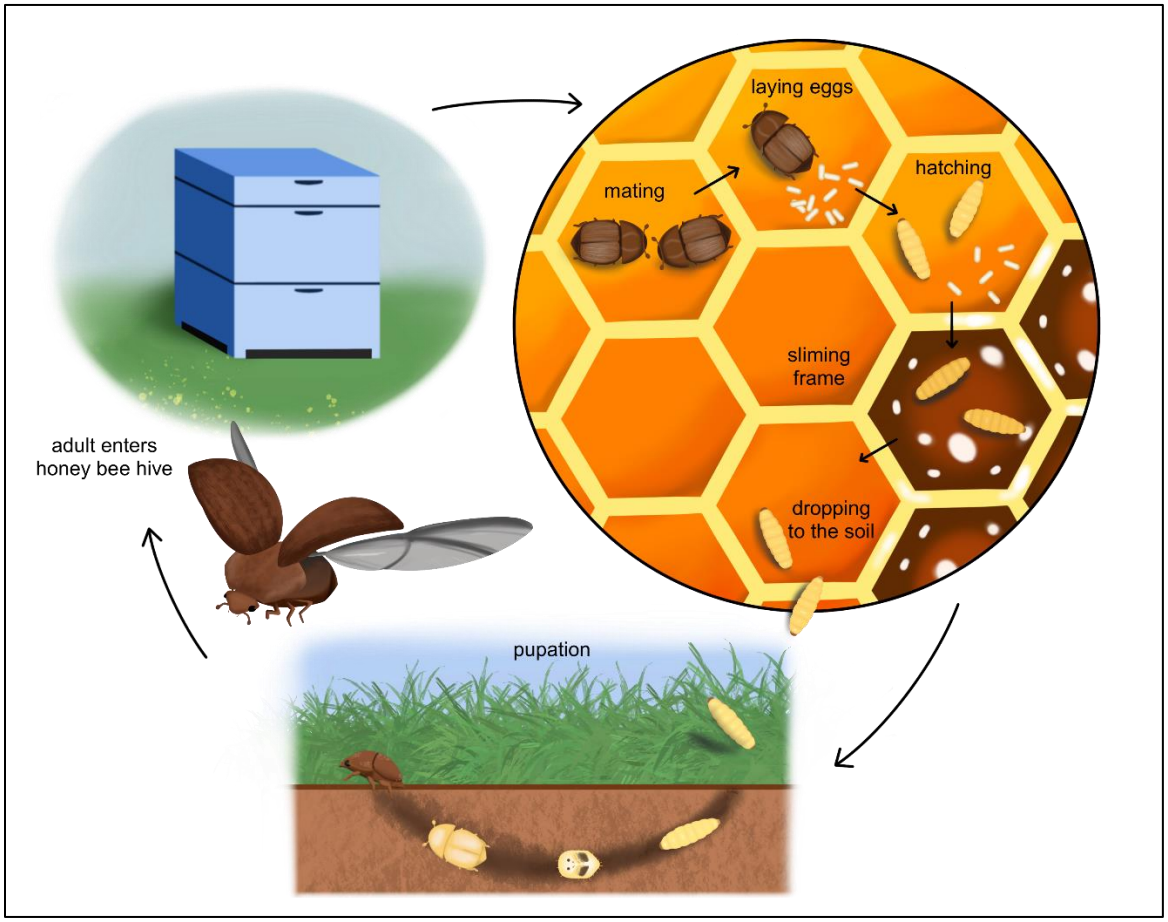
Type	Active ingredients	Tolerant EPN	+ effected EPN[†]	- effected EPN[‡]
Insecticide	Acephate	*S. carpocapsae*		
	Azadirachta indica			*S. feltiae, S. kraussei, H. bacteriophora*
	Beauveria bassiana		*H. indica*	
	Carbofuran			*S. feltiae, S. carpocapsae*
	Carbosulfan	*S. carpocapsae*		
	Cartap hydrochloride			*S. carpocapsae, H. indica*
	Chlorpyrifos	*S. carpocapsae*		
	Cypermenthrin	*S. carpocapsae*		
	Diazinon			*S. carpocapsae*
	Diafenthiuron	*S. feltiae*		*H. bacteriophora*
	Dichlorvos			*S. carpocapsae*
	Ethofenprox	*S. carpocapsae*		
	fenamiphos			*S. feltiae*
	Fenitrothion	*S. carpocapsae*		
	Fenobucarb	*S. carpocapsae*		
	fenoxycarb			*S. feltiae, S. carpocapsae*
	Fenthion			*S. carpocapsae*
	Fenvalerate	*S. carpocapsae*		
	imidacloprid	*S. carpocapsae*		
	Kinoprene	*S. feltiae*		*H. bacteriophora*
	Lufenuron	*S. feltiae*		
	Malathion			*S. carpocapsae*
	Meterhizium anisopliae		*H. indica*	
	methomyl	*S. feltiae, S. carpocapsae*		*H. bacteriophora*
	Mentolcarb	*S. carpocapsae*		
	metoxyfenozide	*S. feltiae*		
	monocrotophos			*H. indica*
	Oxamyl	*S. feltiae, S. carpocapsae*		
	Permethrin	*S. carpocapsae*		
	piperonyl-butoxide	*S. feltiae*		
	profenofos			*S. carpocapsae*
	propetamphos			*S. carpocapsae*
	Proproxur	*S. carpocapsae*		
	prothiofos			*S. carpocapsae*
	pyraclofos			*S. carpocapsae*
	pyriproxyfen	*S. feltiae*		*H. bacteriophora*
	Sulprofos	*S. carpocapsae*		
	tebufenpyrad	*S. feltiae*		
	tebufenozide			*H. bacteriophora*

	temephos			*S. carpocapsae*
	trichlorfon			*S. carpocapsae*
	Trichoderma viride		*H. indica*	
	Xylylcarb	*S. carpocapsae*		
Acaricide	Abamectin	*H. bacteriophora*		
	azocyclotin	*S. feltiae*		
	clofentezin	*S. feltiae*		
	diafenthiuron	*S. feltiae*		*H. bacteriophora*
	Etoxazole	*S. feltiae*		
	fenbutatinoxide	*S. feltiae*		
	fenpyroximate	*S. feltiae*, *H. bacteriophora*		
	Pyrethrin	*H. bacteriophora*		
	tebufenpyrad	*S. feltiae*		
Fungicide	Captan	*S. feltiae*		
	Carbendazium 12% + Mancozeb 3%			*H. indica*
	fenhexamid	*S. feltiae*		
	kresoxim-methyl	*S. feltiae*		
	Nuarimol	*S. feltiae*		*H. bacteriophora*
	Tricyclazole			*H. indica*
Herbicide	Pendimethalin			*H. indica*
	Pseudomonas fluorescens		*H. indica*	

† EPN species that experience increased efficiacy with the addition of the active ingredient in an insecticide, acaricide, fungicide, or herbicide to their environment.

‡ EPN species that experience decreased efficacy with the addition of the active ingredient in an insecticide, acaricide, fungicide, or herbicide to their environment.

Figure 1: Life cycle of *Aethina tumida* Murray, small hive beetle (Veronica Hughes, Hughes



Scientific Illustrations).

Figure 2: (A) Wax frames from *Apis mellifera* colonies damaged by small hive beetle larva (James D. Ellis, University of Florida, Bugwood.org). (B) Adult small hive beetle on wax frames with *Apis mellifera* adults (Jessica Louque, Smithers Viscient, Bugwood.org).

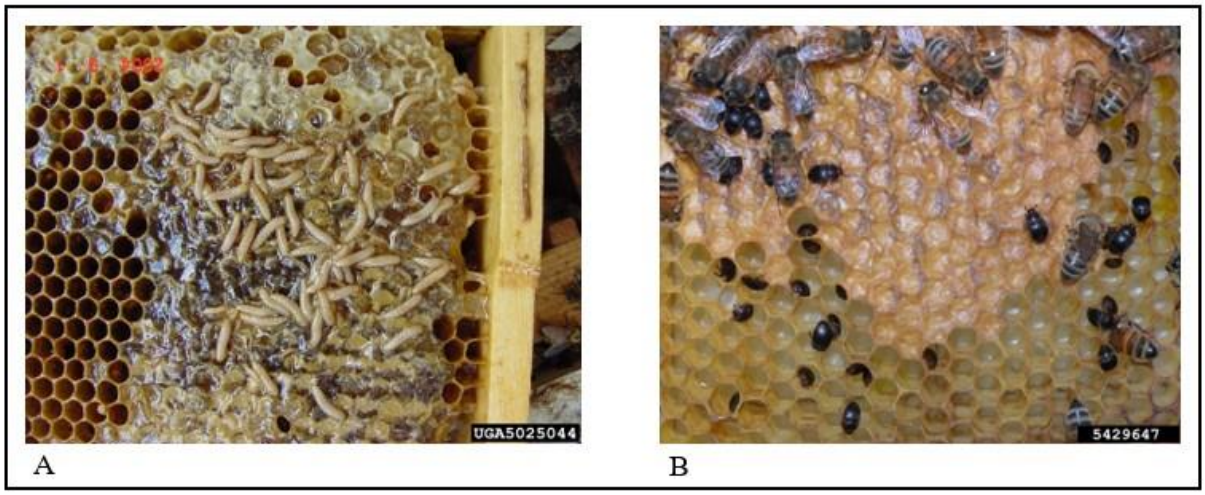
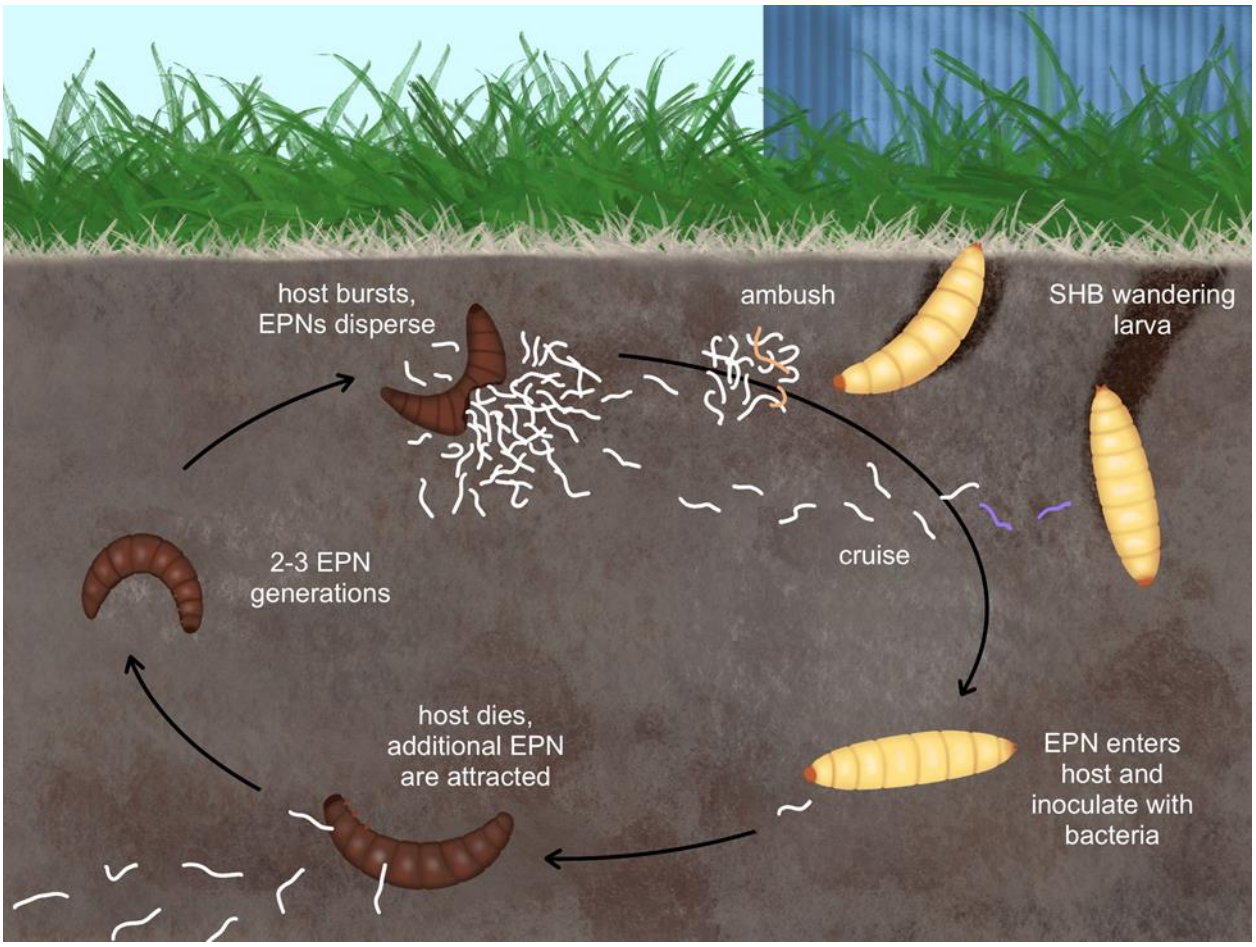


Figure 3: General infection cycle of *Steinernema* spp. and *Heterorhabditis* spp. (Veronica Hughes, Hughes Scientific Illustrations).



Chapter 2: Entomopathogenic nematode management of small hive beetles (*Aethina tumida*) in three native Alabama soils under low moisture conditions

Abstract

The overall goal of this work was to determine the efficacy of entomopathogenic nematodes (EPNs) on *Aethina tumida* Murray (Coleoptera:Nitidulidae) small hive beetle (SHB) in different soil types under low moisture conditions to improve current integrated pest management practices. The objectives were to 1) determine the pupation success of SHB wandering larvae in natural non-autoclaved and sterile autoclaved soil; 2) determine the efficacy of EPNs on SHB wandering larvae in natural non-autoclaved and autoclaved soil in low moisture conditions; and 3) determine the efficacy of EPNs on SHB wandering larvae in three natural non-autoclaved soil types at low moisture levels. The Alabama soils we tested were Kalmia loamy sand (KLS), Benndale fine sandy loam (BFSL), and Decatur silt loam (DSL). For this work, commercially purchased *Heterorhabditis bacteriophora* Poinar, *Steinernema feltiae* Filipjev, and *Steinernema kraussei* Steiner, as well as commercially purchased and laboratory reared *Heterorhabditis indica* Poinar, Karunaka & David, *Steinernema carpocapsae* Weiser, and *Steinernema riobrave* Cabanillas, Poinar & Raulston were tested. We evaluated population densities of 5%, 10%, 20%, 40%, and 80% third stage infective EPN juveniles (IJ3) per 130cc soil. In objective one, pupation success in SHB population densities of 5, 10, and 20 wandering larvae per Petri dish in KLS were similar. Thus, for objectives two and three we used a SHB population density of 5 SHB wandering larvae per Petri dish. Objective two evaluated six commercially purchased and laboratory reared EPN species in natural non-autoclaved and autoclaved KLS soil. Of the six commercially purchased species, *S. carpocapsae* achieved the highest efficacy across all EPN population densities and in both natural non-autoclaved and autoclaved soil with the 69.4% and 84.1% efficacy, respectively.

Steinernema riobrave and *H. indica* achieved the next highest efficacies, however, they were significantly less effective that *S. carpocapsae.* Of the laboratory reared EPNs, the highest efficacy for *S. carpocapsae, S. riobrave,* and *H. indica* was achieved at the population density of 80% IJ3 per 130cc soil. *Steinernema carpocapsae* parasitized 86.7% SHB wandering larvae across all population densities tested. The third objective included all three soil types at the moisture content of 50% field capacity for each soil. At the highest population density, *S. carpocapsae* achieved the best efficacy in KLS, BFSL, and DSL soils at 94.0%, 80.0%, and 47.0%, respectively. In all low moisture EPN experiments, efficacy of each EPN species on SHB wandering larvae was improved when higher EPN population densities were applied. In conclusion, this work suggests that *S. carpocapsae* could be a promising biological control agent to implement into an integrated pest management system for control of SHB in Alabama during low moisture conditions.

Introduction

Apis mellifera honey bees are pollinators that support crop growth and environmental health globally. Their pollination services, along with sales of hive products, makes *A. mellifera* management a billion-dollar industry (Mortensen, 2013; Smith et al., 2013). Pests of *A. mellifera* colonies can negatively affect overall colony health, hive products, and productivity. One such pest is *Aethina tumida,* small hive beetle, a secondary opportunistic pest that completes most of its lifecycle inside bee colonies and pupates in the soil under or around colonies (Zawislak, 2014; Willcox et al., 2017). Infestation of an *A. mellifera* colony begins with adult *A. tumida* flying to a suitable hive, mating, and laying eggs in clusters within the wax frames (Ellis, 2004; Neumann and Elzen, 2004; Graham et al., 2011; Mustafa et al., 2015). The larvae develop by consuming pollen, honey, and *A. mellifera* brood, consequently damaging the frames, fermenting honey, and creating suitable conditions for other pests to thrive (Ellis et al., 2002; Neumann et al., 2013). Larval development speed is dependent on food availability and temperature (Neumann et al.,

2013, 2016). When the larvae are ready to pupate, they drop beneath the hive and begin searching for a suitable pupation location. At this stage, they are called wandering larvae. Once in the soil, *A. tumida* develop into pupae and emerge as adults thirteen to seventy-four days later depending on temperature and soil moisture levels (Neumann et al., 2013).

Control measures for *A. tumida* have included maintaining strong *A. mellifera* colonies, breeding *A. mellifera* for hygienic behavior, monitoring hives for adults and larvae, removing damaged frames, purchasing and baiting *A. tumida* traps, and chemical treatments in or around infested hives (de Guzman et al., 2001; Ellis et al., 2003; Ellis et al., 2004a; Ellis, 2012; Cuthbertson et al., 2013, Smith et al., 2013; Zawislak, 2014, Neumann et al., 2016). Integrated Pest Management (IPM) systems are currently the best control option for SHB control because chemical controls can affect *A. mellifera* individuals as well as hive products (Berry et al., 2013; Fulton et al., 2019).

Recent laboratory bioassay studies suggest that entomopathogenic nematodes (EPN) can successfully infect, feed on, and reproduce in *A. tumida* wandering larva and pupa (Cabanillas and Elzen, 2006; Ellis et al., 2010; Shapiro-Ilan et al., 2010; Cuthbertson et al., 2012; Hill et al., 2016). Subsequently, these EPNs may represent an efficient biological control option for an IPM program. EPNs naturally live in soil and require an insect host to reproduce. The two main genera of EPN that have been marketed for control of *A. tumida* in Europe and North America are *Steinernema* spp. and *Heterorhabditis* spp. These EPN genera have different hunting styles and each species have different environmental and host preferences (Shapiro-Ilan et al., 2002). *Steinernema* spp. generally hunt insect hosts using ambush (sit and wait) techniques while *Heterorhabditis* spp. hunt using cruising (seek and attack) techniques (Lewis et al., 1992; Ellis et al., 2010; Wilson et al., 2012).

Six EPN species that have shown promise in laboratory bioassays for controlling *A. tumida* are *Heterorhabditis bacteriophora, Heterorhabditis indica, Steinernema carpocapsae Steinernema feltiae, Steinernema kraussei,* and *Steinernema riobrave* (Cabanillas and Elzen, 2006; Ellis et al., 2010; Shapiro-Ilan et al., 2010; Cuthbertson et al., 2012; Hill et al., 2016). Infection of *A. tumida* wandering larva occurs when the EPN IJ3 enters the host through a natural orifice and releases a symbiotic gram-negative bacterium that lives within the EPN into the insect hosts hemocoel (Akhurst, 1982; Kaya and Gaugler, 1993; Boemare et al., 1996; Adams and Nguyen, 2002; Stock, 2019). Each of the EPN species mentioned above have a different symbiotic bacterium species that is responsible for killing the host and creating a suitable environment for EPN reproduction (Boemare et al., 1993). The EPNs produce two to three generations within the host cadaver before the new IJ3s leave the cadaver in search of a new host (Smart, 1995).

One limitation of EPNs as biological control agents is the effect of abiotic soil factors on efficacy. Soil particle size, available moisture, temperature, salinity, organic material content, and pH have all been found to affect EPN efficacy to varying degrees (Molyneux and Bedding, 1984; Kung et al., 1990a; Kung et al., 1990b; Kung et al., 1991; Koppenhöfer et al., 1995; Koppenhöfer and Fuzy, 2006; Divya and Sankar, 2009; Kaspi et al., 2010; Shapiro-Ilan et al., 2011; Tofangsazi et al., 2012). Furthermore, soil conditions have also been shown to affect *A. tumida* pupation success (Ellis et al., 2004b; Neumann et al., 2013, 2016). The majority of bioassays screening these EPNs for *A. tumida* management used soil that was sterilized by autoclaving because this removes all living biota from the soil and limits confounding variables within the study. However, autoclaving the soil removes natural biotic competition from the soil and alters the physical and chemical properties so that conditions are not field-realistic. To date,

there is little knowledge about how EPNs perform with natural competition in field soils with varing textures and limited available moisture. Introducing natural soil factors in laboratory bioassays is the next step towards field trials.

The main objective of this research is to determine the efficacy of EPN to control *A. tumida* wandering larva in three soil types found within the state of Alabama at low moisture levels. Specifically the objectives were to 1) determine the pupation success of SHB wandering larvae in natural non-autoclaved and sterile autoclaved soil; 2) determine the efficacy of EPNs on SHB wandering larvae in natural and autoclaved soil in low moisture conditions; and 3) determine the efficacy of EPNs on SHB wandering larvae in three natural non-autoclaved soil types at low moisture levels. Adding EPNs to an IPM system for SHB may benefit beekeepers on a local, state, and national level, improve the health of *A. mellifera* colonies, and subsequent pollination rates in locations where SHB exist.

Materials and Methods

Aethina tumida colony

Approximately 132 male and female SHB adults were field collected in September 2018 via a mouth-operated insect aspirator from ten active honey bee hives placed at the Auburn University (AU) Bee Lab, Auburn, AL. Infected *A. mellifera* colonies selected had not been used for chemical research. Adult SHB were sexed and placed into breeding jars based on protocol described in volume two of the COLOSS Beebook (Neumann et al. 2013). Each breeding jar was labeled and placed in incubators maintained at 25^{o}C, 80% relative humidity (RH), in total darkness (Neuman et al. 2013). Adult and subsequent larvae in the breeding jars were provided a diet of 400g Ultra Bee artificially bee pollen substitute purchased (Mann Lake, Hackensack, Minnesota) to consume and lay eggs on weekly (Neumann et al., 2013). Mature larvae were

placed in plastic pupation jars filled with ~1.75 L sterilized soil that were placed in an incubatore at 25^{o}C, 80% relative humidity (RH), in total darkness for 20 days (Neumann et al., 2013). Increasing genetic diversity in laboratory colonies is important to decrease the chances of inbreeding as well as decrease the potential for genetic branch between wild SHB and laboratory reared SHB. Genetic diversity was promoted in two ways. First, all wandering larvae were combined in a large bin before being placed into a pupation jar. Second, once emerged, adults were sexed and randomly placed into new breeding jars. Only the wandering larvae and pupae phase of the SHB lifecycle were utilized for the study.

Entomopathogenic nematodes

Nematode genera were selected based on previous literature and current market availability. For this study, commercially purchased *Steinernema feltiae* Filipjev, *Steinernema kraussei* Steiner, and *Heterorhabditis bacteriophora* Poinar, *Steinernema riobrave* Cabanillas, Poinar & Raulston, *Steinernema carpocapsae* Weiser, and *Heterorhabditis indica* Poinar, Karunaka & David third stage infective juveniles (IJ3) were tested. We also tested *S. riobrave, S. S. carpocapsae,* and *H. indica* IJ3 reared by the Dr. Shapiro-Ilan, USDA, in Byron, Georgia. EPNs were kept in a standard refrigerator at 4^{o}C until they were needed for each experiment. All experiments were set up within 72 hours of EPN arrival. EPN IJ populations were prepared by placing a 75µm mesh sieve on top of a 25µm mesh sieve and running water indirectly through the sieves. Contents in the 25µm sieve were collected into a glass beaker, diluted to 100 mL with water, and kept at room temperature. The sieves were triple rinsed thoroughly between each nematode species. Each EPN species were enumerated via a Nikon TSX 100 inverted microscope at 40-x magnification. For this study, we used five population density levels: 5-, 10-,

20-, 40- and 80% IJ3 per 130cc soil following methods by Vega et al. (1995) and Ellis et al. (2010). These equated to approximately 121, 243, 485, 971, and 1941 IJ3s per 1mL inoculum.

Soil

The soils used were collected from Auburn University research centers and consisted of a Kalmia loamy sand (KLS) (80% sand, 10% silt, 10% clay) from the Plant Breeding Unit in Tallassee, AL, a Benndale fine sandy loam (BFSL) (73% sand, 20% silt, 7% clay) collected from the Brewton Agricultural Research Unit in Brewton, AL, and a Decatur silt loam (DSL) (24% sand, 49% silt, 28% clay) from the Tennessee Valley Research and Extension Unit near Belle Mina, AL (Table 1). KLS represents the middle of the fine sand to heavy clay soil spectrum we tested to determine the efficacy of EPNs in the wide range of Alabama soils, therefore, KLS was used as the standard for all experiments. Each soil was kept in autoclave bags in a walk-in refreigerator set at 4^{o}C until needed. Half of each of the soils was autoclaved three times at 121^{o}C for 60 minutes with 24 hours between sterilizations (Wolf and Skipper, 1994; Trevors, 1996; Bennett et al., 2003). For the 24 hours between sterilizations, each bag was placed on a laboratory counter to cool to room temperature. After autoclaving, the soils were weighed and then placed in an oven at 38^{o}C (Soil Survey Staff, 2011). Weight was checked every 24 hours until containers were no longer losing weight in accordance with Susha et al. (2014). The dry soil was placed in a new autoclave bag, sealed, and placed back into the walk-in cold room. Non-autoclaved soils were then weighed, placed into the oven at 38^{o}C, and checked as described above. Once prepared, 130g of each soil was placed into a 25mm x 100mm Petri dish. The appropriate amount of moisture was mixed into each soil depending on objective before soils were placed back into the Petri dishes and weighed again. The second weight documented became the standard weight for each soil type.

Experiment 1 - Pupation success of small hive beetle wandering larvae in Kalmia loamy sand

For this experiment, we wanted to to determine the optimim success of SHB wandering larvae at five different concentraions in natural non-autoclaved and sterile autoclaved KLS in order to set a control standard for future experiments. A total of 2,250 wandering larvae of the same age and generation were collected from the SHB colony and split equally into three groups. Soil condition consisted of natural non-autoclaved soil and sterilized autoclaved soil which was prepared at 15% moisture by weight (Neuman et al. 2013). Concentrations of 0, 5, 10, 20, and 40 SHB wandering larvae were evaluated. This experiment consisted of two soil conditions X five SHB wandering larvae densities X five replications X three repeated experiments. In total, 150 experimental units were evaluated. Soil condition consisted of natural non-autoclaved soil and sterilized autoclaved soil at 15% moisture by weight (Neumann et al., 2013). Concentrations of 0, 5, 10, 20, and 40 SHB wandering larvae were added to 130 g of the respective soil and allowed three minutes to burrow. A piece of filter paper was then placed on top of the soil, covered with the Petri dish lid and sealed with parafilm (Ellis et al., 2010). After 24 and 48 hours respectively, the next sets of fifty petri dishes were prepared. All Petri dishes were placed upside down and stored in an incubator at 25^{o}C, 80% relative humidity (RH), in total darkness for 20 days (Ellis et al., 2010; Neuman et al. 2013). To control the effects of confounding variables, each replication was placed in two stacks separated by soil condition (autoclaved or non-autoclaved) and then a random number generator determined the order each of the five units in both stacks should be placed. All five units in both the autoclaved and non-autoclaved stacks for each replication were placed in the same order. Each replication stack was then spaced evenly throughout the incubator in plastic tubs using a randomized complete block design (RCBD) with

each tub containing one block. After 20 days, the contents of each Petri dish were shaken into a bin and each SHB was accounted for. SHB were each documented as a live larva, pupa, or adult, or a dead larva, pupa, or adult. Percent mortality was calculated by dividing the total number of dead larvae, pupae, and adults over the total population of SHB in each dish. Percent success pupation was calculated by dividing the total number of alive adults by the total SHB population in each dish. The SHB concentration that had the most successful emergence percentage was used for the remaining objectives.

Experiment 2 – Entomopathogenic nematode efficacy of small hive beetle wandering larvae in Kalmia loamy sand

For this experiment we wanted to determine optimal efficacy of six commercially purchased EPN species at six population density levels in natural non-autoclaved and sterile autoclaved KLS soil. Commercially available EPN, *Steinernema feltiae, S. riobrave, S. kraussei, S. carpocapsae, H. bacteriophora,* and *H. indica* were purchased to determine which had the best efficacy for SHB. Six population densities of each of the EPN were evaluated and included 0, 121, 243, 485, 971, and 1941 IJ3's per 1mL inoculum the soil. These populations were achieved following methods by Ellis et al. 2010 with 1mL concentrations of 0, 5, 10, 20, 40, and 80% IJ3s per cm^2 soil. This experiment consisted of six EPN species X six EPN concentrations X two soil conditions X five replications and was repeated twice. Petri dishes with soils were set up as described previously. Five SHB wandering larvae were added per Petri dish as previously determined. Petri dishes inoculated with a population of 0 received 1mL of water. Soil was inoculated equally in five locations in the Petri dish – the center, 0^o, 90^o, 180^o, and 270^o approximately one inch away from the edge of the Petri dish. Inoculum was added to the soil, filter paper applied, the lid was secured, and placed in the incubator as previously described. This

experiment was designed as a split-plot RCBD, with EPN species type as the whole plot, and soil condition as the subplot. After 10 days, SHB were recovered as previously described and dissected under a Stereo microscope at 40x for visual confirmation of nematode parasitism (Ellis et al. 2010).

The second part of this experiment focused on the most promising EPN species and concentrations, and soil condition. Laboratory reared *S. carpocapsae, S. riobrave,* and *H. indica* were tested at concentrations of 0, 10, 40, and 80% IJs per cm^2 soil in natural non-autoclaved KSL soil. This experiment evaluated three EPN species X four EPN concentrations X five replications and was repeated twice. For moisture content, we added 50% field capacity of KLS to the prepared soil. The moisture content measurement needed to change for this experiment because the final objective used three soils instead of one and moisture added by percent by weight will not provide an equal amount of soil moisture available between soil particles for EPNs to facilitate movement. This test was constructed and incubated and after 10 days SHB were evaluated as previously described.

Experiment 3 – Entomopathogenic nematode efficacy of small hive beetle wandering larvae in Kalmia loamy sand, Benndale fine sandy loam, and Decature silt loam

To determine if soil type changed the efficacy of EPNs on SHB, three soil types were evaluated. Kalmia loamy sand (KLS) (80% sand, 10% silt, 10% clay), Benndale fine sandy loam (BFSL) (73% sand, 20% silt, 7% clay), and Decatur silt loam (DSL) (24% sand, 49% silt, 28% clay) were selected and placed at 50% field capacity moisture level. Each 25mm x 100mm Petri dish contained one of the three soil types, five SHB wandering larvae, and *S. carpocapsae, S. riobrave,* or *H. indica* at concentrations of either 0, 10, or 80% IJ per cm^2 soil. The experimental unit and method for controlling confounding variables was the same as described previously.

This experiment had a split-split-plot within the RCBD where blocks contained nine stacks separated by replication, then soil type, and finally by EPN species. Visual confirmation and documentation of nematode parasitization of each SHB was performed as previously described.

Data analysis

All data were analyzed in SAS software (Version 9.4, SAS Institute, INC, Cary, NC) using PROC GLIMMIX. Response data from repeated tests were were combined where no interactions were found between repeated trials. Treatment LS-means were separated by Tukey-Kramer at the significance level of $P \leq 0.05$. Standard error of the mean (SEM) was calculated for each parameter mean.

Results

Experiment 1 - Pupation success of small hive beetle wandering larvae in Kalmia loamy sand

The interaction between SHB population density (first factor), and natural non-autoclaved or autoclaved KLS soil (second factor) was not significant (P>0.0993) (Table 2). SHB emergence results indicate that the soil condition of natural or autoclaved did affect pupation significantly. SHB emergence was 64% in autoclaved soil and 71% emergence in the natural non-autoclaved soil (Figure 1). Successful pupation of the SHB decreased by 41.7% (P<0.001), with overall pupation success ranging from 96.0% at the lowest level of 5 wandering larvae and 56.0% at the highest level of 40 wandering larvae per Petri dish (Figure 2). A population density of 5 SHB wandering larvae per was statistically similar to using population densities of 10 or 20 larvae per petri dish, thus this population levels was utilitzed in the following experiments.

Experiment 2 – Entomopathogenic nematode efficacy of small hive beetle wandering larvae in Kalmia loamy sand

The test to determine the efficacy of the six EPN species (first factor) and five EPN populations levels (second factor) in the autoclaved and non-autoclaved soil (third factor) showed no significant interaction between all three factors at $P>0.0652$ (Table 3). Interactions were observed between EPN species and EPN population densities ($P<0.0001$), and EPN species and soil treatment ($P<0.0001$). In sterile autoclaved soil, EPN treatment efficacy of all six EPN species individually with combined data for all five population densities ranged from 84.1% to 5.4% (Figure 3). In natural non-autoclaved soil, EPN treatment efficacy ranged from 69.4% to 2.0%. *Steinernema feltiae, S. riobrave, S. kraussei, H. bacteriophora,* and *H. indica* all obtained statistically similar efficacy, with no significant difference between soil condition ($P>0.1887$) (Table 3). *Steinernema carpocapsae* obtained higher efficacy than all other species tested in both the sterile autoclaved and natural non-autoclaved soils ($P<0.0001$) and across all five-population densities ($P<0.0001$). At each of the five population densities, parasitization success was significantly different between all six EPN species ($P<0.0001$). Percent parasitization of SHB wandering larvae at the five EPN population densities varied between 54.5% and 95.0% (Figure 4). The population density levels of 80% and 40% were effective in parasitizing 94% of the SHB wandering larvae. The 20% population density was effective in parasitizing 78% SHB wandering larvae followed by the 10% and 5% population densities, which were both similar in parasitizing 58% SHB larvae. *Steinernema riobrave* efficacy was highest at 80% population density, *S. kraussei* efficacy was highest at 40% population density, and effiacay for all other EPN species tested was not significantly increased with higher population densities. Based on the results from

this experiment, *S. carpocapsae, S. riobrave,* and *H. indica* at population densities of 10%, 40%, and 80% were selected for further testing.

Further confirming the optimum EPN species and population density, the test observing a two-way interaction between laboratory reared *S. carpocapsae, S. riobrave,* and *H. indica* (first factor) at three population densities (second factor) in non-autoclaved KLS showed no significant interaction ($P>0.4604$) (Table 4). Overall parasitization of SHB wandering larvae varied by 78.4% across the three EPN species with *S. carpocapsae* being more efficient at parasitization (Figure 5). Efficacy of all EPN species increased 38.4% with increasing population density of at 10% to 80% (Figure 6). The EPN population density with the highest percent efficacy of 61.7% occurring at the highest population density of 80%. Based on the results from this experiment, EPN efficacy in KLS at 50% field capacity is greatest when inoculated at the higher population density of 80%. For this reason, the remaining experiment continued to observe efficacy of *S. carpocapsae, S. riobrave,* and *H. indica* at population densities of 10% and 80%.

Experiment 3 – Entomopathogenic nematode efficacy of small hive beetle wandering larvae in Kalamia loamy sand, Benndale fine sandy loam, and Decature silt loam

Expanding the test to include varing soil types, a three-way interaction test between three EPN species (first factor), three soil types (second factor), and two EPN population densities (third factor) showed no significant interaction at ($P>0.1930$) (Table 5). There was a significant two-way interaction between the three EPN species and the three soil types at ($P>0.0003$), as well as the three EPN species and two EPN population densities at ($P<0.0016$). *Steinernema carpocapsae* continued to be the most effective EPN to infect SHB when the soil types were expanded to include the BFSL and DSL soil. Across the varied soil types, *S. carpocapsae*

obtained the highest parasitization with 94.0% in KLS, 80.0% BFSL, and 47.0% in DSL (Figure 7). *Steinernema riobrave* EPN obtained highest parasitization rates of 57.0% in BFSL soil which was 28.8% lower than *S. carpocapsae* in BFSL but the highest efficacy overall for the *S. riobrave* EPN species. *Heterorhabditis indica* EPN had similar parasitim rates in the KLS and BFSL sandy soils and was least parasitic in the DSL clay soil. Efficacy across soil types varied by 84.8% ($P<0.0001$) in KLS, 74.4% ($P<0.0001$) in BFSL, and 85.3% ($P=0.0025$) in DSL (Table 5). Population density did affect EPN parasitism when testing the high and low levels. *Steinernema carpocapsae* was most efficacious of the three species and parasitized more SHB larve at the higher population density of 80% than the lower 10% level (Figure 8). *Steinernema riobrave* followed a similar pattern to *S. carpocapsae*, parasitizing more SBH at the higher 80% level than the 10%. *Steinernema riobrave* was more parasitic than *H. indica* but was less parasitic than *S. carpocapsae*. *Heterorhabditis indica* was equally pathogenic at both population densities. At the 10% and 80% EPN population density, *S. carpocapsae* obtained the highest percent parasitization of SHB larvae. Parasitization increased from 10% IJ3 population density to 80% IJ3 population density by 35.8% for *S. riobrave* and 57.1% for *S. carpocapsae* (Table 5). Parasitization decreased by 3.6% for *H. indica* from the 10% IJ3 population density to 80% IJ3 population density (Table 5). *Steinernema carpocapsae* obtained the highest percent parasitization across all three soils and at both population densities.

Discussion

Biological control agents that can control a pest of *A. mellifera* colonies while not harming *A. mellifera* individuals are important to consider as a part of an integrated pest management program for control of SHB. Based on previous liturature, all six EPN species used in this study had potential as biological control agents for SHB wandering larvae in Europe or

North America. These previous studies were mainly conducted using sterilized sand and various EPN innoculation methods. Many also standardized soil moisture levels as a percentage of water based on weight of the soil. This method of calculating soil moisture does not translate to various natural soil types. In our study, we confirmed EPN efficacy on SHB wandering larvae in various Alabama soil types using field capacity to standardize low soil moisture conditions and bridge the gap between laboratory bioassays and future field bioassays.

Results for our first experiment observing survival rates of SHB wandering larvae at various population densities supported the use of five larvae per Petri dish. Similar studies also used five larvae per dish, however their Petri dish sizes were ~154 cm^2 smaller than what we used (Vega et al.1995; Ellis et al. 2010). Our results concluded that lower SHB population densities do not significantly impact SHB survival rates, therefore, the use of five SHB larvae per Petri dish is adequate. Consequently, this allowed us to optimize experimental units and replications as less resources were utilized. Small hive beetle wandering larvae are not known to pupate in congregations, may travel away from the soil directly beneath a hive in search for a suitable pupation location, and generally pupate in the top 20cm of soil (Frake and Tubbs, 2009; Neumann et al. 2013). For this reason, we used larger Petri dish to better simulate SHB dispersal observed in the field. Based on our results and previous findings, five SHB larvae should be an adequate population density in experiments conducted using materials with an internal space between 42.2cm^3 and 196.3cm^3.

Efficacy varied between the six EPN species and population densities in the initial tests using KLS. Of the six EPN species we tested, only *S. carpocapsae* at an application rate of 80% IJ3 per Petri dish (~1941 IJ3 per 1mL inoculum) showed promise as a biological control agent. Cuthbertson et al. (2012) observed the effects of dipping SHB wandering larvae directly into

solutions containing *S. carpocapsae, S. kraussei,* and *S. feltia* IJ3, treating sand with EPN solutions before adding SHB wandering larvae, and the effects of subsequential applications of EPNs to sand over time. They found similar success as our studies with *S. carpocapsae* across all three teqniques. Interestingly, *S. kraussei* and *S. feltiae* were ineffective when SHB wandering larvae were directly exposed to them, however, *S. kraussei* achieved 100% efficacy when applied to sand and allowed to soak into the sand before SHB wandering larvae were added (Cuthbertson et al. 2012). This suggests that EPN efficacy may also depend on inoculation method.

The two species of *Heterorhabditis* we tested showed less than 50% parasitization of SHB wandering lavae in every soil type and at every population density they were tested in. Previous bioassays conducted using *H. bacteriaphora* to control SHB wandering larvae in autoclaved soil in Florida showed a lower rate of parasitism which was similar to our results (Ellis et al. 2010). Previous bioassays conducted with *H. indica*, however, had almost 100% efficacy between 9 days and 14 weeks post-inoculation (Ellis et al, 2010). Another study conducted with *H. indica* also indicated high efficacy of SHB wandering larvae over 10 to 15 days (Shapiro-Ilan et al. 2010). The main difference between our experiments and these two studies appears to be the method of inoculation and longevity of *H. indica* in soil post-innoculation. In both studies metioned, *H. indica* performed best when inoculated via an infected cadaver instead of an aqueous solution. At this time there are no field bioassays involving inoculation of *H. indica* infected cadavers in the soil under *A. mellifera* hives. Variability of efficacy between experiments conducted using purchased EPNs verses USDA reared EPNs could be caused by factors such as nematode age at time of inoculation and rearing conditions and methods. Purchased EPN species arrive as a mixture of all juvenile stages and are reared by of third-party laboratories that may use different rearing conditions and methods. EPN species

reared by the USDA were the same age at time of inoculation, IJ3, and experienced the same rearing conditions and methods.

Efficacy varied between *S. carpocapsae, S. riobrave,* and *H. indica* in KLS, BFSL, and DSL soils. Efficacy of *S. carpocapsae* and *S. riobrave* appears to be directly related to increase in population density at time of inoculation. *Steinernema carpocapsae* appeared to be the most effective at all population treatment levels. The success of *S. carpocapsae* as a biological control agent for SHB wandering larvae are similar to results found by Cabanillas and Elzen (2006), Shapiro-Ilan et al. (2010), and Cuthberson et al. (2012). *Steinernema carpocapsae* was also the most effective in all three soil types tested. This suggests that *S. carpocapsae* performs better than *S. riobrave* and *H. indica* under low moisture conditions in the loamy sand, sandy loam, and silt loam found in Alabama. The ability of *S. carpocapsae* to parasitize SHB wandering larvae in low moisture conditions is supported by Kung et al. (1991), Grant and Villani (2003), and Koppenhöfer et al. (1995). Previous studies noted that *S. carpocapsae* can survive for up to 16 weeks in sand, sandy loam, clay loam, and clay (Kung et al. 1990) and prefers to hunt near the soils surface (Moyle and Kaya, 1981; Divya and Sankar, 2009). All three of these characteristics further support the idea that *S. carpocapsae* is a viable biological control agent in Alabama.

In summary, of all six EPNs tested, *S. carpocapsae* had the highest infection rates above 80% after 10 days in three soil types at 50% field capacity. Results confirmed that EPN efficacy significantly differ based on soil texture and composition. *Steinernema carpocapsae* and *S. riobrave* were better able to control SHB at the higher population density levels. Results suggest that *S. carpocapsae,* inoculated at $\geq$80% IJ3s per cm^2 soil, is a promising biological control agent for beekeepers in Alabama with hives on loamy sand, fine sandy loam, or silt loam soils during times of low moisture, which is common in this region. One way for beekeepers in Alabama to

determine when their county is experianceing low moisture conditions is through the National Integrated Drought Information System (Hartman, 2020 https://www.drought.gov/drought/rcc/southeast). This EPN biological control agent has good potential to effectively manage SHB when added to a bee keeping integrated pest management program.

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Tables

Table 1: Chemical properties of non-autoclaved and autoclaved soils used in the study[†]

Unit		Non-autoclaved			Autoclaved		
		DSL	BFSL	KLS	DSL	BFSL	KLS
ppm	Ca	22351	695	409	2085	376	230
	K	283	72	58	271	49	68
	Mg	258	62	174	228	147	72
	P	281	36	28	262	32	20
	Al	393	274	107	312	86	184
	B	1.2	0.4	0.4	1.2	0.4	0.4
	Cu	1.4	1	2.7	1.2	1.5	0.8
	Fe	7	22	26	12	19	21
	Mn	91	36	20	590	86	246
	Na	46	39	38	48	40	36
	Zn	20	4	2.9	15	2.4	2.9
	NO_3-N	11	27	12	7	8	21
	$CaCO_3$	3.8	<1.0	<1.0	2.5	<1.0	<1.0
	Soluble salts	238	238	1428	381	159	254

mmhos/cm	**Electrical conductivity**	0.19	0.19	1.12	0.3	0.12	0.2
%	**N**	0.25	0.07	0.035	0.24	0.029	0.07
%	**C**	2.99	1.38	0.54	3.12	0.5	1.41
%	**Organic Material**	5.1	2.4	0.9	5.4	0.9	2.4
%	**S**	0.031	0.009	0.0073	0.027	0.0066	0.013
%	**Moisture**	0.57	0.93	0.026	0.89	0.26	0.68
cm^3/cm^3	**H_2O availability**	0.16	0.09	0.08	0.16	0.07	0.09
	pH	6.00	4.59	6.94	7.00	6.85	5.29

[†]Soil types include Kalmia loamy sand (KLS) collected from AU Plant Breeding Unit in Tallassee, AL, Benndale fine sandy loam (BFSL) collected from AU Brewton Agricultural Research Unit in Brewton, AL, and Decatur silt loam (DSL) and was analyzed at the Auburn University Soil Testing Laboratory.

Table 2: Pupation percentages of *Aethina tumida*, small hive beetle (SHB), in sterile autoclaved or natural non-autoclaved soil[†] and at four population densities after 20 days.[‡]

Variables		Accuracy		
		F measure	**Degree of freedom (df)**	***P***
Main effects	SHB population density	27.94	200	<0.0001
	Soil condition	5.54	200	0.0195
Interaction effects	SHB population density x Soil condition	2.12	200	0.0993

Soil condition	**% SHB pupation**
Autoclaved	64.0 ± 5.2b
Non-autoclaved	70.8 ± 5.8a
P	0.0193
SHB population density (wandering larvae per Petri dish)	
5	96.0 ± 12.4a
10	91.0 ± 11.7a
20	93.5 ± 12.1a
40	56.0 ± 7.2b
P	< 0.0001

[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means within a column followed by the same letter are not significantly different. Data were presented as Mean ± SEM.

Table 3: Percent parasitization of *Athina tumida*, small hive beetle (SHB), wandering larvae after treatments of six purchased entomopathogenic nematode (EPN) species at five EPN population densities in sterile autoclaved or natural non-autoclaved soil[†] after 10 days.[‡]

		Accuracy		
Variables		**F measure**	**Degree of Freedom (df)**	***P***
Main effects	EPN species	19.94	531	<0.0001
	Soil treatment	6.33	531	0.1887
	EPN Population densities	11.32	531	<0.0001
Interaction effects	EPN species x Soil condition	7.64	531	<0.0001
	EPN species x EPN population densities	3.22	531	<0.0001
	Soil condition x EPN population densities	1.62	531	0.1955
	EPN species x Soil condition x EPN population densities	1.09	531	0.0652

EPN species	**% Parasitization of larvae by soil condition**				
	Autoclaved			**Non-autoclaved**	
H. bacteriophora	5.4 ± 0.8de			5.8 ± 0.8de	
H. indica	5.4 ± 0.8de			2.0 ± 0.3e	
S. carpocapsae	84.1 ±11.9a			69.4 ± 9.8b	
S. feltiae	9.6 ± 1.4cd			5.2 ± 0.7de	
S. kraussei	5.6 ± 0.8de			12.4 ± 1.8cd	
S. riobrave	11.7 ± 1.7cd			16.3 ±2.3c	
P	<0.0001			<0.0001	
	% Parasitization of larvae at EPN population densities				
	5	**10**	**20**	**40**	**80**
H. bacteriophora	1.0 ± 0.2g	7.3 ± 1.6fg	4.3 ± 1.0fg	10.5 ± 2.4efg	5.1 ± 1.1fg
H. indica	3.3 ± 0.7fg	6.0 ± 1.3fg	2.3 ± 0.5fg	4.0 ± 0.9fg	3.0 ± 0.7fg
S. carpocapsae	54.5 ± 12.2c	62.8 ± 14.0c	78.4 ± 17.5b	95.0 ± 21.2a	93.3 ± 20.9a
S. feltiae	5.0 ± 1.1fg	7.0 ± 1.6fg	5.1 ± 1.1fg	7.0 ± 1.6fg	12.9 ± 2.9ef
S. kraussei	6.9 ± 1.5fg	2.5 ± 0.6fg	4.0 ± 0.9fg	26.5 ± 5.9d	5.3 ± 1.2fg
S. riobrave	5.8 ± 1.3fg	7.0 ± 1.6fg	19.9 ± 4.5de	8.0 ± 1.8fg	29.4 ± 6.6d
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$. Means within a column followed by the same letter are not significantly different. Data were presented as Mean ± SEM.

Table 4: Percent parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species at three population densities in non-autoclaved soil[†] at 50% field capacity after 10 days.[‡]

		Accuracy		
Variables		**F measure**	**Degree of Freedom (df)**	***P***
Main effects	EPN species	52.96	64	<0.0001
	EPN Population densities	6.31	64	0.0032
Interaction effects	EPN species x EPN population densities	0.92	64	0.4604
EPN species		**% Parasitization of larvae**		
H. indica		18.7 ± 2.2c		
S. carpocapsae		86.7 ± 10.2a		
S. riobrave		41.2 ± 4.9b		
P		<0.0001		
EPN Population density				
10		38.0 ± 4.3b		
40		46.9 ± 5.3ab		
80		61.7 ± 7.0a		
P		0.0032		

[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$).

Table 5: Percent Parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species at two EPN population densities in three soil types† at 50% field capacity after 10 days.‡

Variables		Accuracy		
		F measure	**Degree of Freedom (df)**	***P***
Main effects	EPN species	88.78	153	<0.0001
	Soil type	16.68	153	<0.0001
	EPN Population densities	19.87	153	<0.0001
Interaction effects	EPN species x soil type	5.69	153	0.0003
	EPN species x EPN population densities	6.70	153	0.0016
	Soil type x EPN population densities	0.61	153	0.5446
	EPN species x Soil type x EPN population densities	1.54	153	0.1930

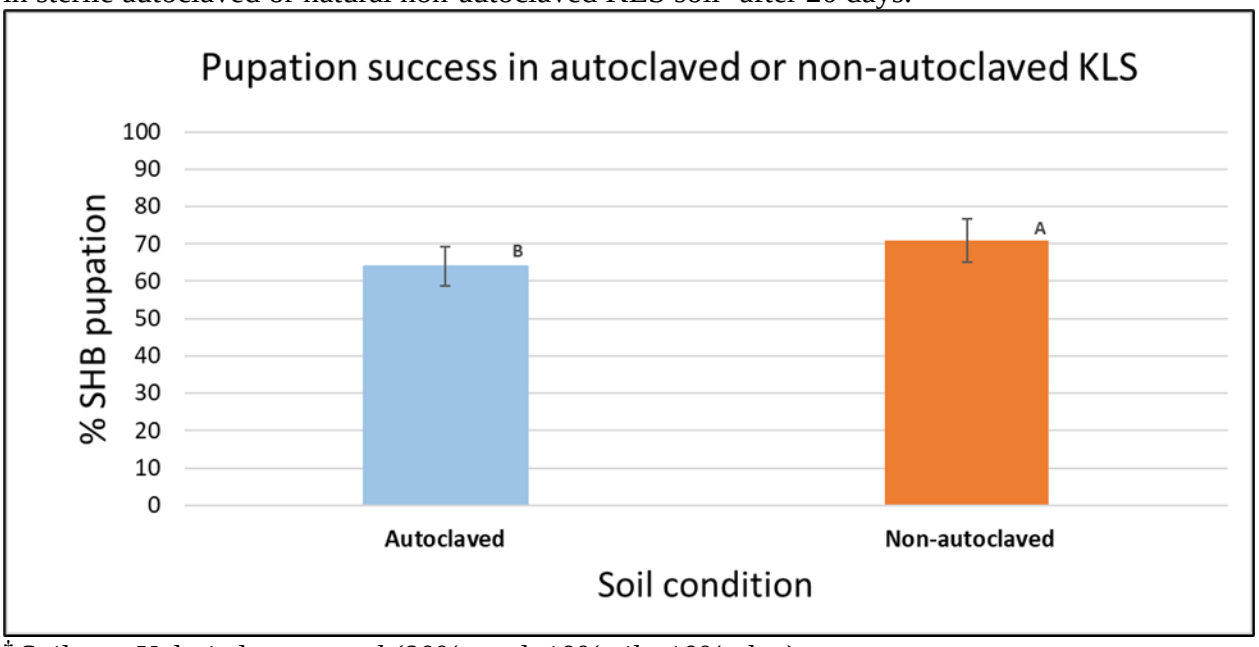
EPN species	**% Parasitization of larvae by soil type**		
	KLS	**BFSL**	**DSL**
H. indica	14.3 ± 1.2ef	20.5 ± 1.7de	5.5 ± 0.44f
S. carpocapsae	94.0 ± 7.6a	80.0 ± 6.5a	47.0 ± 3.8bc
S. riobrave	32.0 ± 2.6cd	57.0 ± 4.6b	30.0 ± 2.4de
P	<0.0001	<0.0001	0.0025
	% Parasitization of larvae by EPN population densities		
	10		**80**
H. indica	13.7 ± 1.6d		13.2 ± 1.6d
S. carpocapsae	57.3 ± 6.8b		90.0 ± 10.6a
S. riobrave	31.0 ± 3.7c		48.3 ± 5.7b
P	<0.0001		<0.0001

† Soil types include Kalmia loamy sand (80% sand, 10% silt, 10% clay) collected from AU Plant Breeding Unit in Tallassee, AL, Benndale fine sandy loam (BFSL) (73% sand, 20% silt, 7% clay) collected from AU Brewton Agricultural Research Unit in Brewton, AL, and Decatur silt loam (DSL) (24% sand, 49% silt, 28% clay).

‡Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$).

Figures

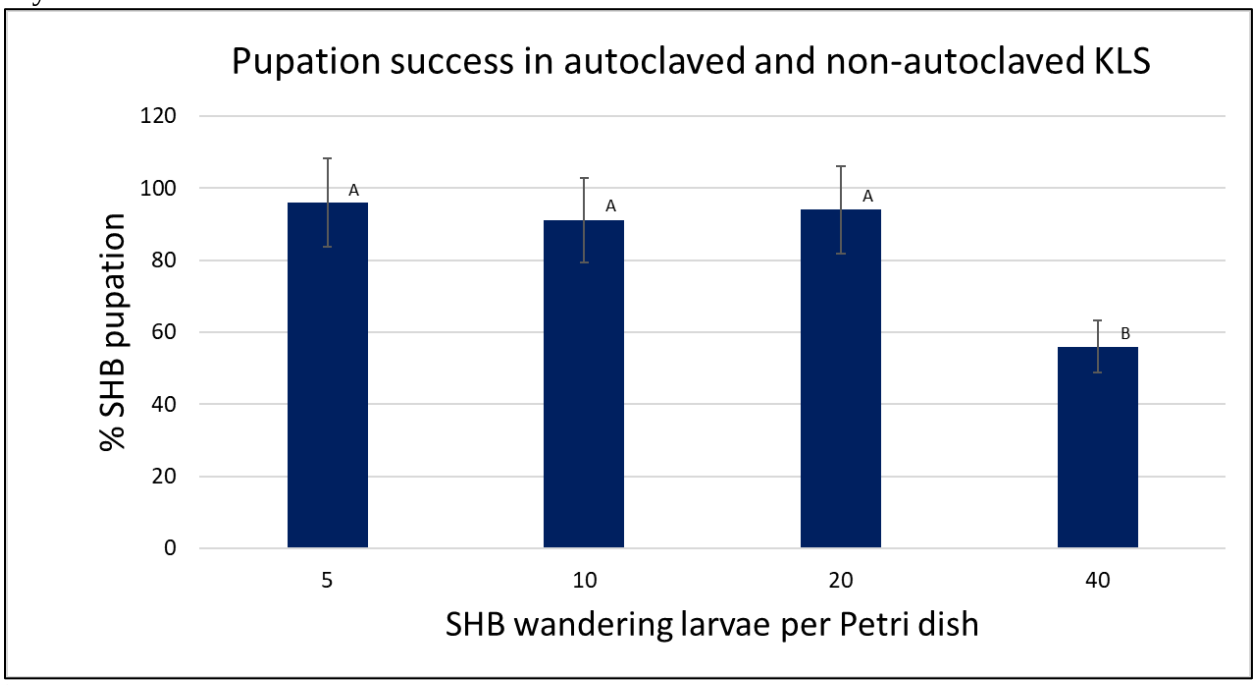
Figure 1: Pupation survival rates of *Aethina tumida*, small hive beetle (SHB), wandering larvae in sterile autoclaved or natural non-autoclaved KLS soil[†] after 20 days. [‡]



[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

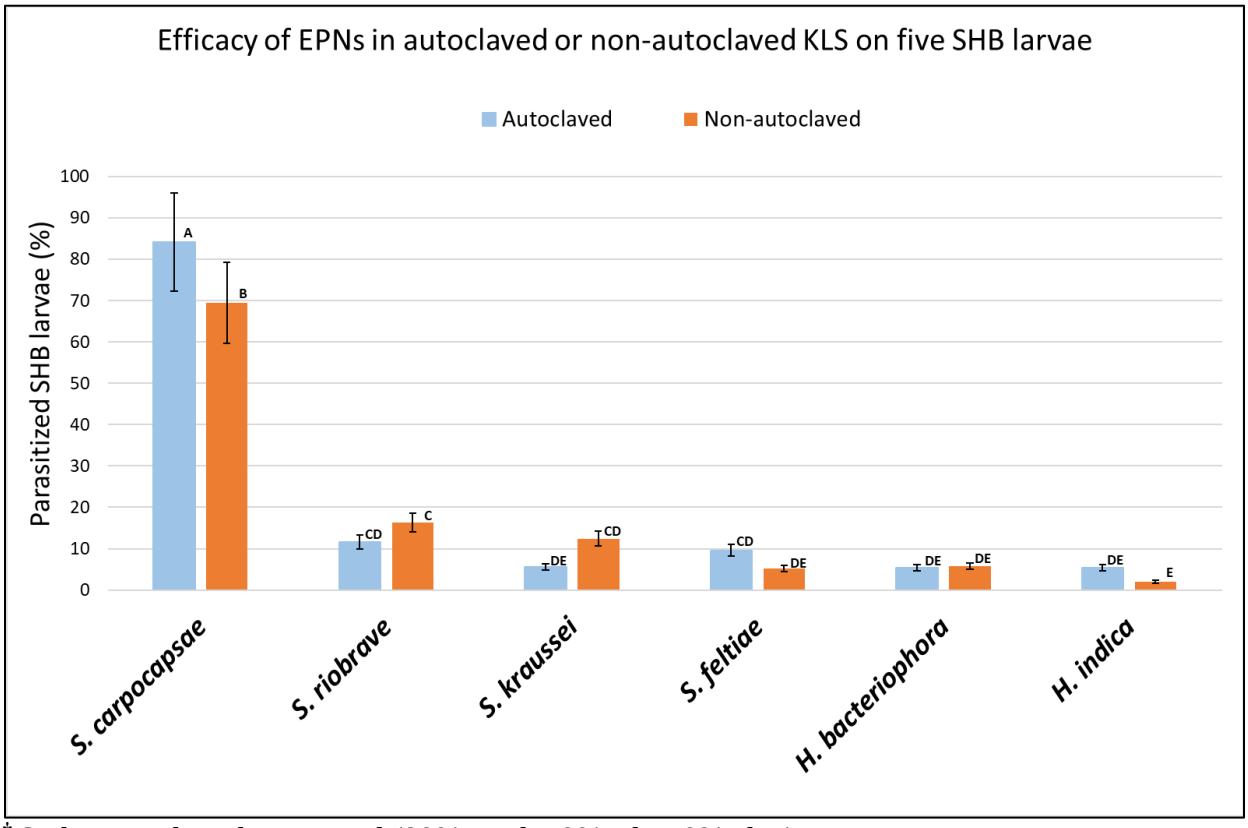
Figure 2: Pupation survival rates of *Aethina tumida*, small hive beetle (SHB), wandering larvae at four population densities in sterile autoclaved and natural non-autoclaved KLS soil[†] after 20 days.[‡]



[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

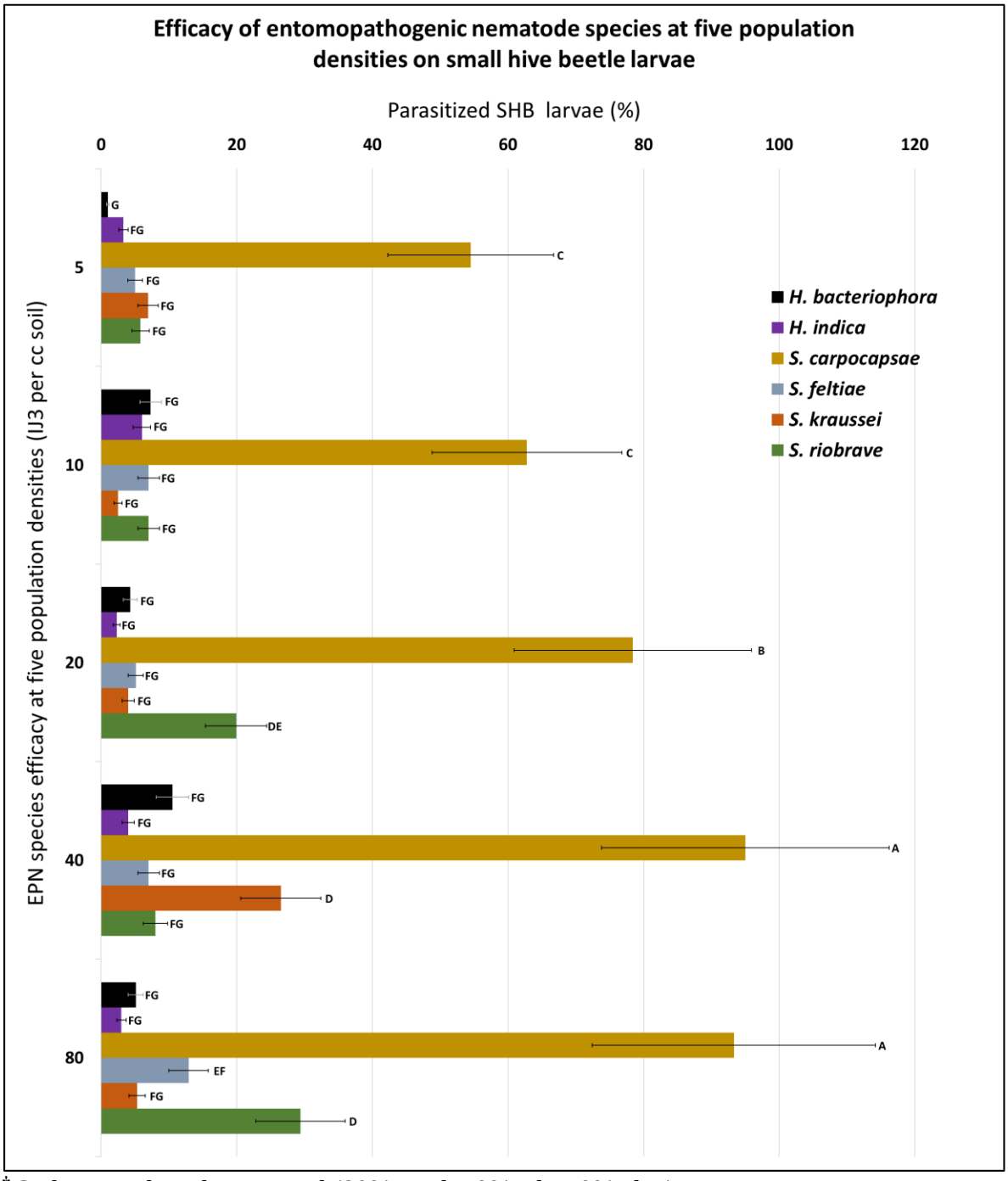
Figure 3: Percent parasitization of *Athina tumida*, small hive beetle (SHB), wandering larvae after treatments of six purchased entomopathogenic nematode (EPN) species in sterile autoclaved or natural non-autoclaved KLS soil[†] after 10 days.[‡]



[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

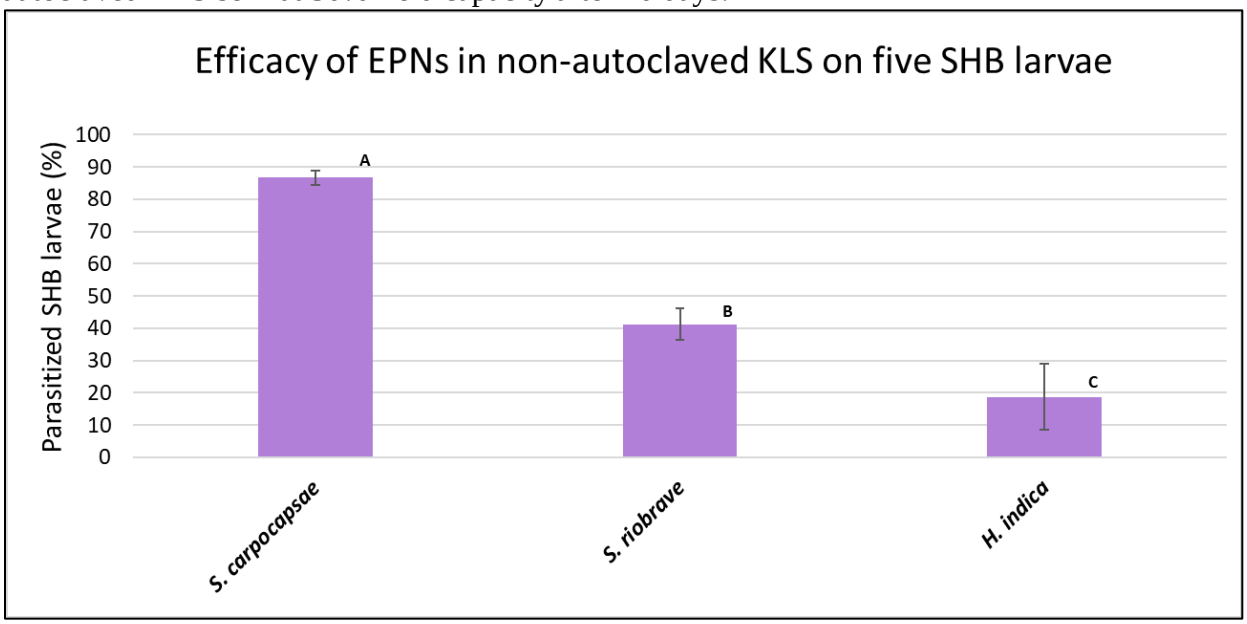
Figure 4: Percent parasitization of *Athina tumida*, small hive beetle (SHB), wandering larvae after treatments of six purchased entomopathogenic nematode (EPN) species at five EPN population densities in sterile autoclaved and natural non-autoclaved KLS soil[†] after 10 days.[‡]



[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

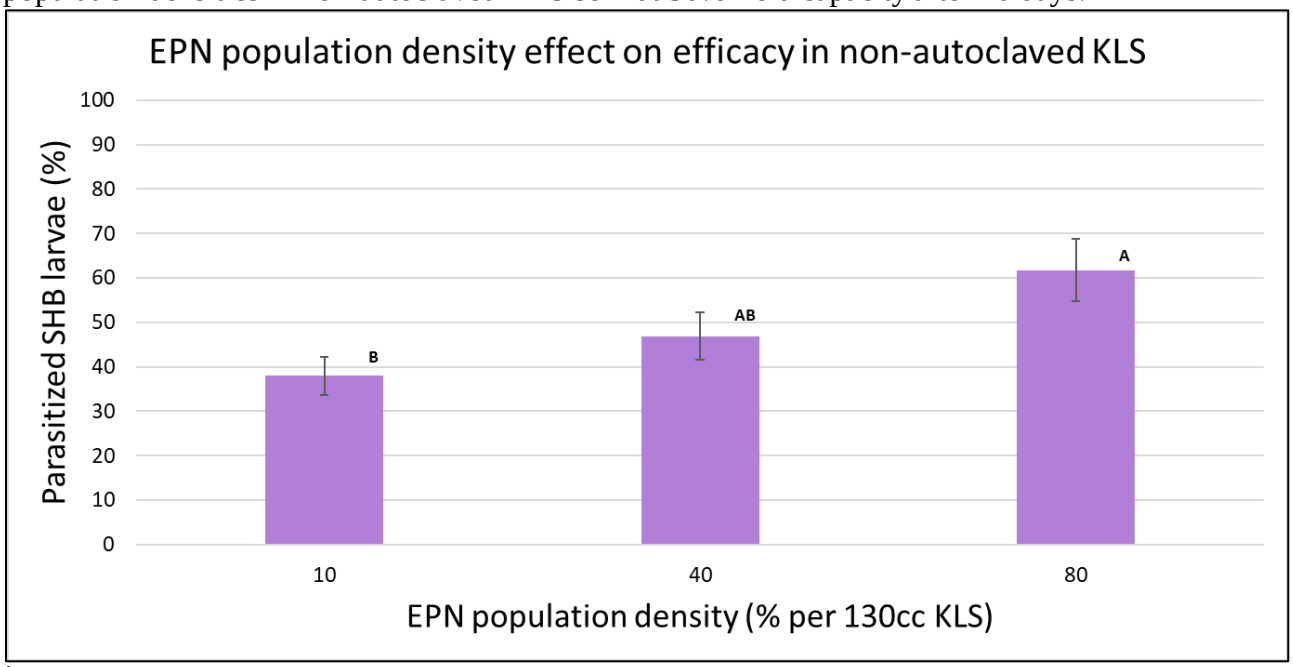
Figure 5: Percent parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species in non-autoclaved KLS soil† at 50% field capacity after 10 days.‡



† Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

‡ Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

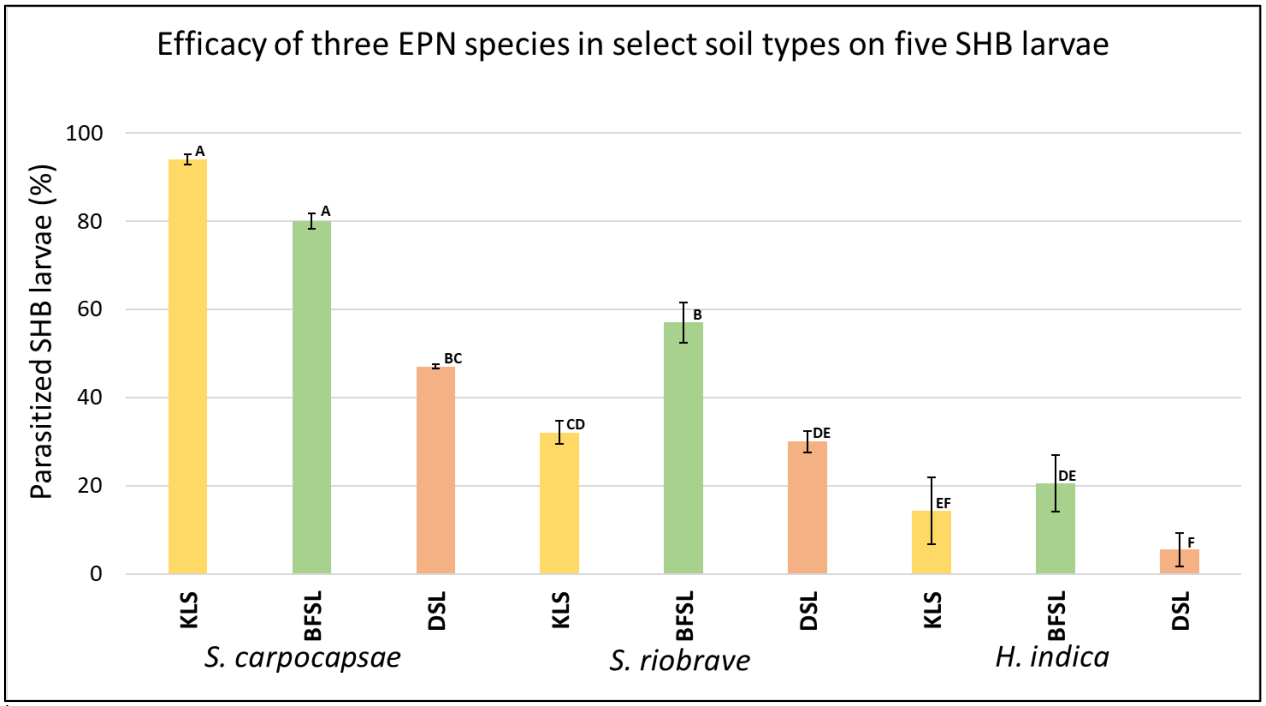
Figure 6: Percent parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species at three population densities in non-autoclaved KLS soil[†] at 50% field capacity after 10 days.[‡]



[†] Soil was Kalmia loamy sand (80% sand, 10% silt, 10% clay).

[‡] Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

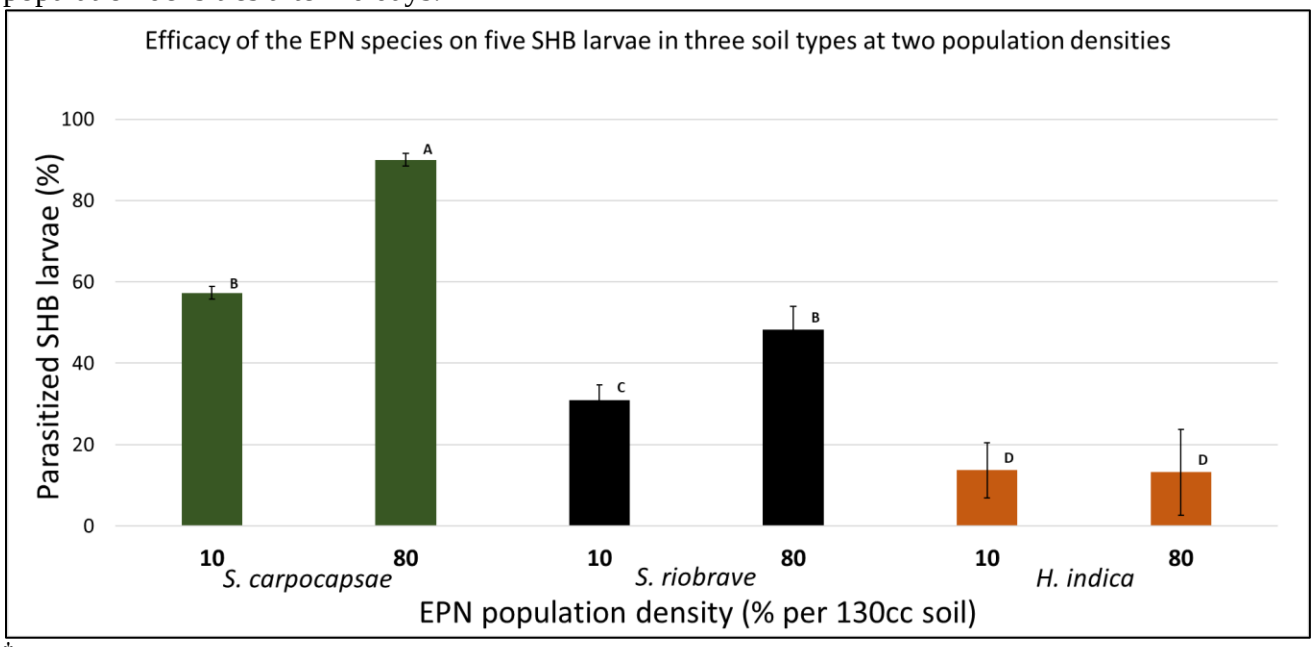
Figure 7: Percent Parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species in three soil types† at 50% field capacity after 10 days.‡



† Soil types include Kalmia loamy sand (80% sand, 10% silt, 10% clay) collected from AU Plant Breeding Unit in Tallassee, AL, Benndale fine sandy loam (BFSL) (73% sand, 20% silt, 7% clay) collected from AU Brewton Agricultural Research Unit in Brewton, AL, and Decatur silt loam (DSL) (24% sand, 49% silt, 28% clay).

‡ Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.

Figure 8: Percent Parasitization of *Aethina tumida*, small hive beetle (SHB), wandering larvae after treatments of three USDA reared entomopathogenic nematode (EPN) species at two EPN population densities after 10 days. †



† Data were analyzed using PROC GLIMMIX and LSMEANS separated by Tukey-Kramer ($P \leq 0.05$). Means followed by the same letter are not significantly different. Error bars are presented as Mean ± SEM.