EFFECTS OF HIGH TEMPERATURE EXPOSURE ON THE SURVIVAL AND INFECTIVITY OF COMMERCIALLY AVAILABLE ENTOMOPATHOGENIC NEMATODES

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Anna Rebecca Giesmann

December 2019

ABSTRACT

Entomopathogenic nematodes (EPNs) are widely used as biological control agents against soil-dwelling insect pests. Three commercially available species are *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) (Hb), *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) (Sc), and *Steinernema feltiae* Filipjev (Sf). This study evaluated the effects of high temperature exposure on both survival and infectivity of these EPNs. Survival was assessed after diluting Hb, Sc, and Sf in water and treating for 1 to 10 h in glass vials in incubators at 30 to 45°C. Overall, Sc was the most heat tolerant, then Hb, then Sf. Treatments at 30°C had no impact on survival of any species, there was varying survival at 35 and 40°C, and treatments of 1 or 2 h at 45°C resulted in complete mortality. Product formulation was not found to have a consistent, significant effect on survival. Both Sc and Sf were treated at 30, 35, and 40°C, then two surviving infective juveniles were applied to individual *Galleria mellonella* Linn. (Lepidoptera: Pyralidae) larvae in well plates filled with moist sand. Larval mortality was assessed after 3 days. Doses of 5000 infective juveniles of Sc and Sf were also applied to moist growing mix in plastic cups, treated at 35 and 40°C, then 15 fungus gnat (*Bradysia impatiens* Johannsen; Diptera: Sciaridae; FG) larvae were added to each cup. Emerging adult FG were caught on sticky card traps and counted 2 weeks after larvae were added. Treatment at 30°C had no impact on Sc or Sf infectivity. Treatment at 35°C did not reduce infectivity of Sc, but infectivity of Sf against both hosts was reduced after 4 and 8 h exposures. After just 1 h exposure at 40°C Sf did not cause any infection, while 2 h treatment slightly reduced Sc infectivity against *G. mellonella*, and 4 h significantly reduced Sc infectivity against FG. Without high temperature exposure Sf caused higher infection of FG than Sc did, so growers may want to rely on Sf unless soil temperatures surpass 4 h at 35°C, or if they reach 40°C for any duration, in which case Hb or Sc may be more effective.

BIOGRAPHICAL SKETCH

Anna was raised in the small, beautiful town of Bethany, WV, by her parents John and Susan Giesmann. She and her brother Matthew spent most of their childhood playing outside, as they explored the woods, hills, and creek behind their house. Anna received a secondary education at The Linsly School in Wheeling, WV, and in 2015 earned a B.S. in Biology from Grove City College in Grove City, PA. After several internships in plant health management at public gardens, she came to Cornell University to further solidify her understanding of pest management. She is committed to helping others appreciate nature, whether it be through formal horticulture and environmental education, or through personal interactions. Anna is motivated by her loving family and friends, and by her faith in Jesus Christ.

ACKNOWLEDGMENTS

Many thanks to those who contributed to the completion of this thesis: Priscilla Thompson, Antonio Testa, Liza White, Heather Grab, Lynn Johnson at the Cornell Statistical Consulting Unit, and of course, the members of my advisory committee, John Sanderson, Elson Shields, and Kyle Wickings. I am also grateful to the Cornell Department of Entomology as a whole, for bringing together so many supportive, enthusiastic students and faculty, and for providing the opportunity for me to learn through both extension and teaching assistantships. EPN products for the experiments in this thesis were generously donated by BASF, E-nema, and Koppert Biological Systems. The work was supported in part by the NY Farm Viability Institute and the USDA Multistate Project NE-1332.

TABLE OF CONTENTS

CHAPTER 1

ENTOMOPATHOGENIC NEMATODES: A REVIEW OF HIGH TEMPERATURE TOLERANCE

General Background

Biological control of pests has existed for millennia, and in modern times it has become its own scientific field and commercial industry. The earliest record of deliberate biocontrol dates to Ancient China, with the distribution of predatory ants to protect citrus. In the 1800's, the use of parasitoids and predators to protect crops was implemented first in Europe, and then North America (van den Bosch 1982). The success of biocontrol programs continued to increase in the twentieth century, apart from a temporary decline in the 1940's (van den Bosch 1982). While microorganisms and arthropods make up a large portion of biocontrol agents, entomopathogenic nematodes (EPNs), or roundworms of the families Heterorhabditidae and Steinernematidae that associate with insect-killing bacteria, also play an important role in many pest management programs (Lewis and Clarke 2012). EPNs are now used across agriculture, horticulture, and even forestry, but to ensure that pests are successfully controlled, management decisions must take into account all aspects of EPN biology (Georgis et al. 2006).

The first description of an EPN, *Aplectana kraussei* Steiner (now *Steinernema kraussei*), was in 1923, but it was not until they were detected in codling moth larvae in the 1950s that intensive research began. Morphological studies in the 1960s and new methods to culture EPNs in the 1970s greatly increased research potential (Poinar and Grewal 2012). Since then, researchers across continents have elucidated much of EPN biology, and developed more efficient modes of production and application. Publications on EPNs increased from an average of 24 articles per year in the 1980s, to 73 per year in the 1990s, to 91 per year in the 2010s (San-Blas 2013). While biocontrol and behavior have remained the most common topics of EPN research, studies on their symbiotic interaction with bacteria, relationships with other organisms, and methods for mass production have increased (San-Blas 2013).

As primarily soil-dwelling organisms, EPNs are usually applied to control the cryptic immature life stages of pests. There are limited observations of EPNs' natural host range, but they are successfully used against numerous dipteran, coleopteran, hymenopteran, and lepidopteran pest species (Lewis and Clarke 2012). Although they have six life stages, including the egg, four juvenile stages, and the adult, only the third juvenile stage can live outside the host and move through the soil to find a new host. This free-living stage is also referred to as the infective juvenile, or IJ (Lewis and Clarke 2012). While searching, IJs respond to insect-emitted odorants such as CO_2, as well as many other sensory cues. Aside from these cues, EPN species have a range of general foraging strategies, on a continuum from sit-and-wait "ambushers," to actively searching "cruisers" (Gang and Hallem 2016). Most EPNs have also been observed to forage in groups, which would give them an advantage in overcoming a host's immune response (Shapiro-Ilan et al. 2014).

After locating an acceptable host, the IJ enters through its spiracles, mouth, or anus, then penetrates the tracheole or gut wall to enter the hemocoel. Once inside, the nematode releases its symbiotic bacteria to break down the insect's tissues, as well as to help suppress insect immune response (Dowd and Peters 2002). This bacterial release begins the "recovery" phase of IJs, as they exit diapause, shed their cuticles, and become adults (Lewis and Clarke 2012). The nematodes feed on both the decomposed host tissues and the multiplying bacteria, and produce two to three generations. When resources become depleted, a new generation of IJs will exit the cadaver, and begin searching for a new host (Kaya and Gaugler 1993).

While this general life cycle applies to all EPNs, there are some marked differences between the two genera most commonly used for biocontrol, *Heterorhabditis* and *Steinernema*. First, although foraging strategy exists on a continuum, *Heterorhabditis* IJs tend to actively seek hosts as cruisers, while *Steinernema* IJs are more often ambushers. In the infection stage, *Heterorhabditis* have a proximal tooth to assist in entry, while *Steinernema* lack this structure (Lewis et al. 2006). Immediately after infection,

Heterorhabditis must shed both their own cuticle and the retained second stage juvenile cuticle referred to as the "sheath." *Steinernema* IJs initially retain the sheath, but lose it more easily during foraging, leaving them less protected from environmental and predatory risks (Campbell and Gaugler 1991). During bacterial release into the hemocoel, *Heterorhabditis* nematodes regurgitate *Photorhabdus* symbionts which had been stored throughout the gut, while *Steinernema* nematodes defecate *Xenorhabdus* symbionts which had been stored in a special vesicle within the gut (Ciche and Ensign 2003, Snyder et al. 2007). Finally, during reproduction, the first generation of adult *Heterorhabditis* are always hermaphroditic, while all *Steinernema* except *S. hermaphroditum* are amphimictic (Lewis and Clarke 2012).

Before 1980, fewer than 10 species of EPNs had been described, but as research increased, surveys were carried out worldwide to find native species and strains, and now around 100 species have been described (San-Blas 2013, Peters et al. 2016). Newly isolated nematodes are often designated as a strain of a previously described species. However, these isolates are not always fully described, and therefore may lack shared characteristics within the group, or be a distinct, undescribed species. Several common EPN species have been reassigned or made synonymous in the past, which can result in confusion in the literature (Kaya and Gaugler 1993). To ameliorate this problem, in the 1990s the European Cooperation in Science and Technology created a working group of nematologists from 17 different countries to share knowledge within the growing field. They collaborated for several years before issuing protocols for EPN identification, criteria for describing new species, and a list of accepted species (Hominick et al. 1997).

Currently, most mass-rearing of EPNs is focused on *Heterorhabditis bacteriophora* Poinar, *Steinernema carpocapsae* Weiser, and *Steinernema feltiae* Filipjev, although several other species are produced for specific markets (Kaya et al. 2006). EPN production can occur in vivo, but this is generally labor intensive and provides small outputs even when mechanized. EPNs can also be grown in vitro, with

solid-state culture on agar or sponges, or in liquid culture. The latter method has been developed to the point that nematodes are grown in several-ton steel bioreactors, which provide control over conditions like pressure, temperature, dissolved oxygen, and pH (Peters et al. 2016).

When EPNs are produced in these massive quantities and shipped long distances after purchase, it is crucial for them to remain viable until application (Grewal 2000). EPNs can either be mixed with an inert carrier that allows for continued movement, or they can be put into an active carrier, which induces EPNs into a quiescent state, often anhydrobiotic (Grewal et al. 2005). Two of the most common commercial EPN formulations are a polyacrylamide gel or powder-based matrix, though exact components of formulations are proprietary and are rarely disclosed. Other carriers include diatomaceous earth, vermiculite, potting mix, and compost, which have been shown to provide different levels of EPN preservation at varying temperatures (Leite et al. 2018).

Once a pest manager has acquired an appropriate EPN product, there are several application methods from which to choose. Most spray equipment is suitable for applying EPNs, as long as the suspension remains agitated and filters are removed. EPNs can also be applied through irrigation in sprinkler or drip systems (Bateman et al. 2007). With any of these methods, pressure within the system should be maintained below 2000 kPa for *S. carpocapsae*, and 1400 for *H. bacteriophora* to avoid physical stress (Fife et al. 2007). While these options function as drenches onto the soil, it is also possible to apply nematodes under the soil surface, either as an injection, usually in agricultural fields, or within small capsules mixed into in the growing substrate (Hiltpold 2015). For growers who manage their own EPN production, host cadavers can be applied directly to the soil surface, letting IJs emerge naturally and seek out fresh hosts (Gumus et al. 2015). The application of EPNs onto foliage has been met with mixed success, being somewhat effective on whitefly nymphs, but ineffective for all foliar thrips life stages (Buitenhuis and Shipp 2005, Cuthbertson et al. 2007).

After application, many factors can affect EPN success against the intended soil pests. On a biological level, the EPN must have the ability to find the host and infect the life stage that is present (Georgis et al. 2006). Especially in outdoor conditions, diverse soil biota can also impede EPN success through predation, infection, and competition (Helmberger et al. 2017). Abiotic factors affecting EPNs include the soil type, UV radiation, and moisture. Potting mixes with hardwood bark can decompose more slowly, retain more water, and decrease oxygen content, which is less ideal for EPNs than a pine bark mix (Jagdale et al. 2004). Porous mixes with peat, bark, and coir allow for better EPN distribution compared to peat and compost mixes, however, virulence may be unaffected since only a few IJs are needed to infect (Ansari and Butt 2011). High electrical conductivity (EC) and moisture retention in soils can reduce EPN infectivity, while sand content increases it, and pH does not have an effect (Kaspi et al. 2010). Directly after application, or while foraging in the upper soil layer, EPN viability and virulence can be reduced by ultraviolet radiation. This effect varies between EPN species, but the risk may be avoided by applying EPNs early or late in the day (Shapiro-Ilan et al. 2015).

Another abiotic factor which can affect EPNs is temperature. Climate control within greenhouses can sometimes be inefficient and expensive (Cuce et al. 2016), so many businesses must cope with suboptimal production temperatures at certain times of year. This can potentially result in a lapse in pest control, especially after sudden increases in ambient temperature. The effects of high temperature exposure on EPN survival and infectivity have been widely studied, and will be discussed in the following section of this review.

Mechanisms for Impact of High Temperatures on EPNs

While it is widely accepted that high temperature exposure can be a major factor affecting EPN survival and efficacy against pests, the mechanisms are not fully understood. Much research has investigated the role of energy reserves for IJ mortality, how these reserves change with temperature, and additional physiological changes that occur with heat exposure.

Since IJs are a free-living, but non-feeding life stage, they must rely on previously acquired energy reserves to sustain them until finding and penetrating a suitable new host (Van Gundy et al. 1967). Rate of lipid depletion can vary between EPNs, with one study finding that *Heterorhabditis* lost significantly more lipids than *Steinernema* after 12 weeks of storage at 23°C (Menti et al. 2000). When stored in 20°C water, the rate of energy depletion explained 93.8% of the variation in survival time of *H. megditis* Poinar, Jackson, and Klein (Fitters and Griffin 2006). In another study that examined several *Heterorhabditis* strains, *S. carpocapsae,* and *S. riobrave* Cabanillas, Poinar and Raulston stored at temperatures ranging from 8-28°C*,* lipid reserves decreased in all EPNs over time, though significantly more quickly at 24 and 28°C (Andaló et al. 2011).

There is conflicting evidence concerning whether lipid reserves are an accurate indicator of nematode activity and infectivity. Patel and Wright (1997) found that low neutral lipid reserves result in lower infectivity for *S. feltiae, S. glaseri* Steiner*,* and *S. riobravis* (syn. *S. riobrave*), but *S. carpocapsae* can remain infective. Patel et al (1997) suggest that this is due to the utilization of glycogen. Croll and Matthews (1973) studied the ageing of the hookworm *Ancylostoma tubaeformae* Zeder, and although not an EPN, decreasing lipids in the closely-related nematode were not always correlated with a decrease in activity. While *H. megditis* IJs naturally lost energy reserves over time, Fitters and Griffin (2004) observed that those with lower lipid levels are more easily activated, indicating they may take greater risks to penetrate a host when facing starvation. In addition, although Hass et al. (2002) observed a correlation between decreasing energy reserves and the mortality and decreasing infectivity of *Heterorhabditis spp.* IJs, some natural mortality occurred when reserves were still high.

In a study that examined energy reserves of six EPN species, including *S. carpocapsae, S. feltiae,* and *H. bacteriophora*, *S. scapterisci* Nguyen and Smart was found to have the highest saturated fatty acid content, which would aid survival in its native tropical range (Selvan et al. 1993). *S. riobravis* has a higher ratio of saturated to unsaturated fatty acids when maintained at 30°C, compared to 20 or 25°C

(Abu Hatab and Gaugler 1997). Likewise, another study found that *S. carpocapsae* and *S. feltiae* had an increased level of unsaturated fatty acids, compared to saturated, as storage temperatures decreased from 25 to 5°C. However, a *S. riobravis* strain from Texas did not exhibit this modification as temperatures decreased, likely because it is less adapted for cold conditions (Jagdale and Gordon 1997).

Like with most animals, EPNs synthesize heat-shock proteins in response to high temperature exposure. Using PCR and Southern blotting, Hashmi et al. (1997) revealed that five *Heterorhabditis* species had polymorphisms in the heat-shock gene *hsp70*, although a correlation was yet to be drawn between these polymorphisms and a difference in gene products and subsequent thermotolerance. Soon after, Hashmi et al. (1998) examined *H. bacteriophora* with or without additional *hsp70* genes transformed from *Caenorhabditis elegans*, and those with additional *hsp70* exhibited much higher survival after high temperature exposure. When treated at 35°C, *H. bacteriophora* also accumulates trehalose and the enzymes associated with its metabolism, likely to protect against desiccation at extreme temperatures (Jagdale et al. 2005). Solomon et al. (2000) first described Desc47, a protein synthesized at the same time as trehalose by *S. feltiae*, in response to desiccation.

Whatever the mechanisms, high temperatures have been clearly shown to affect EPN biology. Many studies have tried to elucidate these effects, usually focusing on a subset of species or strains, EPN sources, temperature ranges, and durations of exposure. Although a few studies have included commercially produced EPNs, most research has focused on wild isolates. Findings from this body of research will be grouped by the three most commonly used species of EPNs: *H. bacteriophora, S. carpocapsae,* and *S. feltiae*, although some studies have included more than one of these. Research on other species will be discussed in brief, as well. A summary of these studies can be found in Table 1.1.

High Temperature Tolerance of Heterorhabditis bacteriophora *(Hb)*

Hb is among the species whose high temperature tolerance has been studied the most. Overall, it is one of the more heat tolerant of the commercially available EPN species. It has been reported to

survive short exposures to high temperatures, such as 2h exposure to 37°C, and the mean temperature to kill 50% of EPNs (LT_{50}) for various strains ranges from 33.3 to 40.1°C (Morton and García-del-Pino 2009, Mukuka et al. 2010). The range for infectivity, or ability to infect pests, is slightly more restrictive, with the most effective reported range being 24 to 27°C (Shapiro et al. 1999).

Several studies have focused on determining the LT_{50} of Hb populations. Ehlers et al. (2005) pre-exposed a hybrid strain of Hb and eleven descendant inbred strains to 35°C for 3h, allowed recovery at 25°C for 1h, then placed them in a gradient of water baths ranging from 37.6 to 39.8°C. Surviving juveniles were collected and counted, and the mean temperature tolerated was between 38.3 and 39.3°C. Mukuka et al. (2010) used a similar protocol, except 36 natural populations and 18 hybrid lines of Hb were treated, one of which was a commercial strain HY-EN 01, as described by Johnigk et al. (2002). Some nematodes of each population received a 3h pre-exposure at 35°C, then all nematodes were treated for 2h in water baths ranging from 32 to 41°C. The LT_{50} ranged from 33.3 to 40.1°C when not pre-exposed, and 34.8 to 39.2°C when they were pre-exposed. Susurluk and Ulu (2015) isolated Hb from various regions of Turkey, placed aliquots of 500 IJs in individual well plate cells, treated them at 38°C for 2h, then checked mortality. The LT_{50} ranged from 37.4 to 39.0°C among populations, and the LT_{90} ranged from 40.0 to 41.3°C. Although the LT_{50} range for Hb populations determined by Mukuka et al. is a much wider than the range determined by Ehlers et al. (2005) or Susurluk and Ulu (2015), they overlap closely.

Some studies have investigated the impact of a wide range of temperatures and durations of exposure. Morton and García-del-Pino (2009) collected soil samples from throughout Mediterranean regions of Spain and treated Hb isolates at 25, 30, 32, 35, 37, 40 and 42°C for 2, 4, 6, 8, 10 or 12h in petri dishes filled with sand. Nematodes were allowed to recover at 25°C for 1h, then were extracted with a Baermann funnel and checked for mortality. The three Hb isolates had high survival at treatments up to 2h at 37°C, although less than 10% survived exposure for 4h at either 35 or 37°C. Exposure for 4h at 32°C

resulted in approx. 55% survival, but increasing exposure to 12h reduced survival to approx. 15%. Finnegan et al. (1999) tested two European isolates of Hb in Eppendorf tubes of either sea water or distilled water at 20, 32, 33, 34, 35, 36, 37, 38, and 39°C for 1h, and Hb survived all treatments. These studies further confirm that Hb can survive 1 to 2h exposure at 35 to 39°C, and slightly longer exposures at slightly lower temperatures.

The same two studies discussed above also examined the effect of temperature on the infectivity of Hb. Morton and García-del-Pino (2009) tested the same Mediterranean Hb isolates against *Galleria mellonella* Linn., with 50 IJs and one larva on moist sand in tissue culture wells. These were incubated at 5, 8, 10, 15, 20, 25, 28, 30, 32, 35, or 37°C, and checked every 24h for larval mortality. No mortality was observed at 5, 8, 10, or 37°C, but mortality increased up through 25°C, and decreased at 28 and at 30°C, The maximum temperature at which Hb caused infection was 35°C. For a subset of the treatments in Eppendorf tubes, Finnegan et al. (1999) put 10 surviving Hb and one *G. mellonella* larva in petri dishes with sand, and incubated at 20°C. Resulting larval mortality was significantly lower when Hb had been treated at 35°C or higher in distilled water, or at 37°C or higher in sea water. The same isolates were also heat treated with 100 IJs in glass tubes of sand and either water type at 20, 37, or 39°C for 1, 2, 4, 6 or 24h, and allowed to recover. One *G. mellonella* larva was then added to each tube and incubated at 20°C for 2 days. There was much higher larval mortality when Hb had been treated at 20°C in distilled water compared to sea water, approx. 95% and 25%, respectively, but for high temperatures larval mortality was greatly reduced after distilled water treatments and only slightly with sea water treatments, all approx. 20%. This is similar temperature range for Hb infectivity as suggested by Morton and García-del-Pino (2009), and both assert that the range for peak Hb infectivity is 20 to 25°C, resulting in very high *G. mellonella* mortality.

Susurluk and Ulu (2015) also assessed the infectivity of Hb, for strains isolated from Turkey, with or without pre-adaptation to heat for 2h at 38°C. *G. mellonella* were exposed to nematodes in individual

well plate cells, with doses of 1 to 5 IJs per larva. Plates were incubated at 25°C for 4 days, then larvae were assessed. No mortality was observed with a dose of 1 or 2 IJs of any Hb, and the mortality was approximately 5, 25, and 60% for doses of 3, 4, and 5 IJs, respectively. There was no difference in the infectivity of Hb with and without heat adaptation, suggesting that Hb infectivity can be extended to higher temperatures if exposure is short.

Several studies have focused on the effect of prolonged temperature exposure on the infectivity of Hb. Chung et al. (2010) examined of two Korean isolates of Hb, placing 5 to 160 IJs and one *G. mellonella* on moist filter paper in petri dishes. Dishes were incubated at 13, 18, 24, 30, or 35°C, and larval mortality was checked daily. The dose to kill 50% *G. mellonella* decreased for both strains as temperature increased, until the most efficient infection at 24 and 30°C. The time required to kill 50% *G. mellonella* decreased as temperatures increased, until 30°C for both strains, then the time required increased at 35°C. Grewal et al. (1994) examined the effects of a wide range of temperatures on several species of EPNs, including a strain of Hb from Utah, USA. Aliquots of 50 IJs were added to dishes of moist sand and acclimatized to temperatures 8 to 39°C for 2h before adding one *G. mellonella* larva to each dish, which remained at the treatment temperature. Mortality was checked every 8h for up to 20d. The lowest temperature at which Hb caused infection was 10°C, with approx. 20% mortality. Larval mortality increased to approx. 80% at 12, 15, 20, and 32°C, while at 25 and 30°C it surpassed 90%. The quickest rate of infection by Hb occurred at 30°C, when it took only 32h to reach 50% mortality. There was no mortality at 35°C or higher. These results are partially consistent with those from Chung et al., which also indicated quickest infection by Hb at 30°C, but suggested that low infection can occur at 35°C.

Some research has examined Hb infectivity against hosts besides *G. mellonella*. Molyneux (1986) tested a wide range of temperatures with a strain of Hb from New Zealand, against 3rd instar larvae of the sheep blowfly (*Lucilia cuprina* Wiedemann). Individual larvae were exposed to doses of 320 IJs in plastic jars filled with moist sand, and incubated at temperatures from 0 to 40°C, in 5°C increments.

Treatments at 18°C or above were incubated for 10 to 14d, and treatments below were incubated for 28d. Maximum infection was approx. 85% at 25°C, then decreasing to approx. 70% at 30°C, 20% at 35°C, and no infection at 40°C. Examining the effect of milder temperatures, Mahar et al. (2005) tested Hb (HW79 strain) against the larvae and pupae of black vine weevil (*Otiorhynchus sulcatus* Fabricius). One larva or pupa and 100 IJs were placed in individual well plate cells filled with moist sand, then incubated at 20 or 25°C for 2 days. Significantly more IJs penetrated both larval and pupal hosts at 25°C. Shapiro et al. (1999) tested the effects of a similar temperature range on the infectivity of Hb (Hb1 strain, Rutgers University) against sugarcane rootstalk borer beetle (*Diaprepes abbreviatus* Linn.). Approx. 500 IJs were added to moist sand in cups, each containing one buried larva of varying ages. Cups were incubated at 21, 24, or 27°C for 14d, then checked for larval mortality. Mortality was higher for larvae under 50d old (approx. 50 to 60%) compared to those 100d old (approx. 20%). Mortality was lower at 21°C than at either 24 or 27°C, which did not differ. These studies suggest that Hb infectivity against a variety of hosts is highest from 24 to 27°C, when facing prolonged temperature exposure.

High Temperature Tolerance of Steinernema carpocapsae *(Sc)*

Sc is another important EPN for biological control, so its high temperature tolerance has also been the subject of much research. Along with Hb, it is one of the more heat tolerant of the commercially available EPN species. Some research indicates it can survive prolonged exposure to 35°C, and has some survival after short exposures at higher temperatures, such as 2h at 40°C (Kung et al. 1991, Somasekhar et al. 2002). Susurluk and Ulu (2015) included Sc in their study of EPNs from Turkey, and the LT_{50} ranged from 35.5 to 35.7°C among populations, and the LT_{90} ranged from 38.2 to 38.7°C. Infectivity of Sc is sometimes reported to be very high at 35°C, with some infection occurring at 40°C (Kung et al. 1991, Somasekhar et al. 2002). Other reports vary in descriptions of Sc heat tolerance, with Morton and García-del-Pino (2009) observing less tolerance at the same temperatures tested, and Ali et al. (2007) observing some tolerance at temperatures as high as 45°C.

The following four studies examined the effect of high temperatures on both Sc survival and infectivity. Kung et al. (1991) tested the effects of long-term exposure a strain of Sc from former biocontrol company BioSys Inc. (Palo Alto, CA). Approximately 3500 IJs were pipetted into each tube containing moist sandy loam soil, then exposed to 5, 15, 25, or 35°C for durations up to 96 days. After each treatment period, either a centrifuge flotation technique was used to extract surviving nematodes, or contents of the tubes were emptied into a dish, 10 *G. mellonella* larvae added, and incubated at 25°C for one week. Survival of Sc was significantly lower at 35°C than at other temperatures, with approx. 75% survival after 4d, 50% after 16d, 30% after 32d, and only approx. 5% survival after the 96d treatment. Survival of Sc and its infectivity against *G. mellonella* were both highest at 25°C, then 15°C, then 5°C. Approx. 90% *G. mellonella* mortality occurred on samples treated at 35°C for 4d, but then only 70% for 16d, 45% for 32d, and 10% mortality on samples treated for 96d.

Somasekhar et al. (2002) tested the effects of a single temperature and duration, but on one commercial and 14 wild-isolated strains of Sc from across the eastern United States. Approximately 1000 IJs in 1 mL of water were placed in individual well plate cells, exposed to 40°C for 2h. Recovery was allowed for 24h at 25°C, then survival was assessed. After treatment, IJs were also tested for infectivity against *G. mellonella*, placing one IJ and one larva in each cell of well plates, and checking larval mortality after 48 and 72h. Survival of nematodes varied from 37 to 82% between strains, with greater survival in strains isolated from North Carolina than from Ohio. *G. mellonella* mortality varied between 22 and 76% after 48h exposure, and between 42 and 79% after 72h exposure.

Morton and García-del-Pino (2009) included one strain of Sc in their study of Mediterranean EPNs, and it survived up to 4h exposure to 35°C. It did not cause *G. mellonella* mortality at 8 or 10°C, maximum mortality occurred at 20 to 25°C, and some larval mortality occurred at temperatures up to 32°C. These results indicate less heat tolerance than the commercial strain of Sc tested by Kung et al. (1991), or the commercial and wild strains from the USA, tested by Somasekhar et al. (2002).

Ali et al. (2007) tested the effects of a wide range of temperatures on a strain of Sc isolated from Kapur, India. Approx. 1000 IJs were placed in moist, sandy loam soil in clay pots, and incubated for 6h at 15, 20, 25, 30, 35, 40 or 45°C. Surviving nematodes were either extracted with a sieve and Baermann's funnel and counted, or the nematodes were applied to the prepupa of *Helicoverpa armigera* Hübner in pots, incubated at 30°C, and checked for mortality after 3 days. The highest nematode survival was 81% at 25°C, decreasing to approx. 75% at 30°C, 60% at 35°C, 55% at 40°C, and approx. 50% at 45°C. The highest *H. armigera* mortality was 82% at 25°C, decreasing to approx. 80% at 30°C, 60% at 35°C, 50% at 40°C, and approx. 40% at 45°C. This description of the effects of temperature up to 35°C on Sc are similar to reports in other studies, but Ali et al. (2007) suggests that some heat tolerance extends to even higher temperatures, for short exposures up through 45°C.

Other research has focused on the effect of temperature on just the infectivity of *S. carpocapsae*. Grewal et al. (1993) applied 100 IJs of Sc ("All" strain) to petri dishes containing moist sand, then one *G. mellonella* larva was added to each dish. Dishes were exposed to 15, 20, 25, 30, 32.5, 35, or 37.5°C, and mortality was checked every day for 4 days. After the full 4 days at 15°C *G. mellonella* mortality only reached approx. 65%, but it was 100% after 48h at 20°C, 24h at 25°C and after 48h at 30 and 32.5°C. Mortality decreased significantly at 35 and 37.5°C, reaching only approx. 90% and 40%, respectively, after the full 4 days. These results are consistent with the studies previously described, suggesting that the optimal temperature for infection by Sc is 25°C, although some infection occurs at temperatures up through 37.5°C.

Grewal et al. (1994) included two strains of Sc, from Georgia and New Jersey, USA, in their study. The lowest temperature at which *G. mellonella* mortality occurred was 10°C, when mortality was approx. 30% and 60% for the two strains, respectively. Mortality surpassed 90% from 12 to 30°C, decreased to approx. 65% at 32°C, and there was no mortality caused by Sc at 35°C. The quickest rate of infection, in hours required to reach 50% mortality, occurred at 30°C for both strains, with 21h and 24h,

respectively. Overall, these results suggest a slightly lower temperature tolerance for the strains of Sc tested, as the optimal temperature for infection was close to 25°C, but the upper limit for infection was below 35°C.

Grewal et al. (1999) carried out a similar test, except nematodes were only treated at 28°C, and exposed to *G. mellonella* with various bioassay methods. In vitro and in vivo-reared Sc caused approx. 65% *G. mellonella* mortality at 1 IJ per larva after 48h on either moist filter papers or on sand in well plates. This is lower host mortality than reported at similar temperatures in other studies, suggesting that the dose of IJs is also an important factor for infection.

Several studies have examined the effects of a lower range of temperature on Sc infectivity. Saunders and Webster (1999) applied approx. 300 IJs of Sc ("All" strain) to petri dishes lined with moist filter paper, added one *G. mellonella* larva, then exposed the dishes to 8, 12, 16, 20, or 24°C. Larval mortality was checked 4 times a day for 4 days, then twice a day for up to 27 more days. The LT_{50} was 80h at 16°C, 52h at 20°C, and 40h at 24°C. Radova and Trnkova (2010) obtained nematodes from the Academy of Sciences of the Czech Republic, and added either 50 or 500 IJs to small plastic boxes of moist soil. Ten mealworm larvae (*Tenebrio molitor* Linn.) were added to each box, the boxes were incubated at 10, 15 or 25°C, and larval mortality was checked after 5, 7 and 14 days. No *T. molitor* mortality occurred at 10°C until 14d, but after 5d at 15°C mortality was approx. 20% with 50 IJs and approx. 50% with 500 IJs. Mortality was greatest for all treatment durations at 25°C, with approx. 50% and 80% mortality after 5d, 50% and 85% after 7d, and 60% and 95% mortality after 14d, with 50 and 500 IJs, respectively. Mahar et al. (2005) included Sc ("All" strain) in their tests against black vine weevil, and more larval and pupal hosts were infected at 25°C compared to 20°C. These three studies reinforce that 25°C is the optimal temperature for infection by Sc, although slower, lower rates of infection can also occur at lower temperatures.

High Temperature Tolerance of Steinernema feltiae *(Sf)*

Sf is another EPN species commonly used for biocontrol, so many studies have examined its temperature tolerance as well. Reports on the effect of temperature on the survival of Sf varies widely with different strains, with some reports of no survival after 4h at 35°C, and other reports suggesting an LT_{50} of up to 39.7°C (Morton and García-del-Pino 2009, Susurluk and Ulu 2015). Grewal et al. (1994) reported that Sf has a more restrictive high temperature limit for infectivity than either Hb or Sc, with no infection above 30°C, as opposed to 32°C for the other species. However, most research also suggests that Sf has more extreme low temperature limit for infectivity, with infection occurring at 10 or as low as 8°C (Grewal et al. 1994, Morton and García-del-Pino 2009).

A few studies have sought to clarify the mean temperature tolerated by 50% of Sf IJs. Susurluk and Ulu (2015) included Sf in their study of isolates from Turkey, along with Hb and Sc. The LT_{50} for Sf ranged from 35.6 to 36.7°C among populations, and the LT_{90} ranged from 37.3 to 39.0°C. Addis et al. (2011) examined the effects of high temperature on the survival of 4 Indonesian isolates of Sf. First, 200 IJs were added to chambers with 2mL of water, a subset were exposed to 35°C for 3h for heat adaptation, then all were exposed for 2h to temperatures between 37 to 41°C. These were allowed to recover at 25°C overnight, then surviving nematodes were isolated with a water trap and counted. The mean temperature tolerated for non-adapted Sf strains ranged from 38.4 to 38.9°C, and for adapted Sf it was 38.9 to 39.7°C. These results suggest a higher heat tolerance than reported for the strains tested by Susurluk and Ulu (2015).

Morton and García-del-Pino (2009) included 14 Sf isolates in their study of Mediterranean EPNs, along with Hb and Sc. Eleven of the isolates of Sf had low survival (approx. 10%) after 4h at 35°C, and no survival after 12h exposure. These same isolates had approx. 50% survival after 4h at 32°C, which decreased to approx. 20% after 12h. The other three isolates of Sf had no survival at 35°C, and only approx. 10% after 4h treatment at 32°C. The range of survival for the eleven heat tolerant strains of Sf is

similar to the results for Hb and Sc in this study, although the less tolerant strains of Sf indicate varying tolerance between strains, and lower tolerance than Hb or Sc on average. This study suggests less heat tolerance for Sf survival than reported with LT_{50} by Addis et al. (2011) or Susurluk and Ulu (2015). Morton and García-del-Pino (2009) also reported that no *G. mellonella* mortality was observed at 5 or 37°C, but it increased at 8 through 25°C, then decreased at 28 and at 30°C. No Sf was found inside *G. mellonella* treated at 32 and 35°C, but some larvae had been killed, possibly by undetected nematodes. This suggests a lower temperature limit for infectivity by Sf compared to Hb or Sc, as only Sf caused infection of *G. mellonella* at 8 or 10°C. However, like with Hb and Sc, the optimal temperature for infection was still 25°C.

Molyneux (1985) examined the effect of low to mid-range temperatures on the survival and infectivity of Sf ("Agriotos" strain), although at the time it was considered synonymous with *Neoplectana carpocapsae*. Aliquots of 1000 IJs were introduced into jars of moist sand and exposed to 10, 15, 23, or 28°C for 1 to 32 weeks. After treatment, either the sand was sieved and surviving nematodes counted, or a larva of *L. cuprina* was added. Nematode survival decreased to 10 to 15% after just two weeks at 23 and 28°C, and survival was 0% at these temperatures after 24 and 16 weeks, respectively. Survival at 15°C was only slightly higher, with approx. 30% survival after 2 weeks. Survival of Sf only decreased to approx. 60% after 32 weeks at 10°C. By 8 weeks exposure at 23 and 28°C there was no infection of *L. cuprina* by Sf, but after the same duration of exposure there was still 20% infection at 15°C and 70% infection at 10°C. Infection at 15°C never surpassed 10% after 16 weeks exposure, but after 32 weeks at 10°C there was still 20% infection. These results suggest that 10°C is the optimal temperature for both Sf survival and infectivity, if undergoing long-term exposure.

Several studies have examined the effect of temperature on just the infectivity of Sf. Menti et al. (2000) included two strains of Sf in their study, from Greece and the UK. Aliquots of 100 IJs were placed in screwcap tubes of moist sand, one *G. mellonella* larva placed inside, and the tubes were incubated at

10, 15, 18, 23, 30 or 35°C. After 3 days exposure, larval mortality was checked, and nematodes from dissected cadavers were counted. The highest nematode penetration occurred at 23°C, when approx. 35% the nematodes applied were counted in the host. No infection occurred at 10°C, there was an increase in penetration at 15, 18, and 23°C, infection was low at 30°C, and none occurred at 35°C. This is a more restrictive low temperature range for Sf than in studies previously discussed, which had reported infection by Sf at 10°C. With only 3 day exposure, Menti et al. (2000) suggests 23°C is the optimal temperature for both Sf survival and infectivity.

Grewal et al. (1994) included two strains of Sf from Argentina and France in their study, along with Hb, Sc, and others. At 8°C, Sf was the only species to cause *G. mellonella* mortality, approx. 40 to 50%. Mortality then surpassed 90% from 10 to 25°C, decreased to approx. 60% at 30°C, and there was no mortality caused by Sf at 32°C or higher. The quickest rate of infection, by hours required to reach 50% mortality, was 24h at 25°C for the strain from Argentina, and 37.6h at 30°C for the strain from France. This is consistent with reports from Morton and García-del-Pino (2009) and Molyneux (1985) that Sf can infect at 10°C, though Grewal et al. (1994) here suggests that infectivity is the same for temperatures up through 25°C. This study also shows that higher temperatures can result in quicker infection by Sf, even if the percentage of infection is lower overall.

Radova and Trnkova (2010) included Sf in their study of low to mid-temperature infectivity against *T. molitor*. Significantly higher mortality occurred at 10°C with 500 IJs of Sf compared to Sc, after both 7 and 14d. After 5d at 15°C, Sf-caused mortality was approx. 2% with 50 IJs and approx. 45% with 500 IJs. Mortality was greatest for all durations at 25°C, with approx. 10% and 75% mortality after 5d, then rising to 30% and 80% mortality after 14d, with 50 and 500 IJs, respectively. For all durations at 25°C, a dose of 50 Sf IJs resulted in significantly lower mortality than 50 Sc IJs, however, there was no difference between doses of 500 IJs at 25°C. These results suggest that although Sf has higher infectivity at 10°C compared to other EPN species, its infectivity is still higher at 25°C compared to 10 or 15°C.

Mahar et al. (2005) included Sf isolated from UK in their tests against black vine weevil, and significantly more IJs penetrated larval and pupal hosts at 20°C compared to 25°C, which was the opposite of both Hb and Sc.

High Temperature Tolerance of Other EPN Species

Many other species of *Heterorhabditis* and *Steinernema* have been subject of a few experiments, but none of them have been examined exhaustively. Overall, temperature tolerance varies greatly within genera, and even between strains of the same species. The effect of temperature on infectivity has been studied for *H. heliothidis* Khan, Brooks and Hirschmann, *H. indica* Poinar Karunakar, and David, and *H. megidis*, as well as *S. glaseri, S. riobrave,* and *S. scapterisci*, among others, as follows.

H. indica caused higher mortality of *D. abbreviatus* than Hb causes at 24 or 27°C, and caused higher mortality at 25°C compared to 20°C, for the larvae and pupae of *O. sulcatus* (Shapiro et al. 1999, Mahar et al. 2005). A study by Mukuka et al. (2010) observed that with an LT_{50} of 33.3°C, a strain of *H. megidis* had lower tolerance than any strains of Hb or *H. indica* tested. The latter two species had a range of LT_{50} values from 34.7 to 40.1°C, interspersed between species, and there was no significant difference between the species overall. In other research, *H. megidis* was shown to have a lower heat tolerance than *S. riobrave* or *S. glaseri*, as its infectivity against *G. mellonella* decreased significantly starting at 30°C (Grewal et al. 1994). Menti et al (2000) compared *H. megditis* to Sf with strains isolated from Greece and the UK, and neither species infected *G. mellonella* at 30 or 35°C. Finally, *H. megditis* strain H90 was compared to Sc "All" strain, and both had higher infectivity at 20 and 24°C compared to lower temperatures, and their rates of infection did not differ at those temperatures (Saunders and Webster 1999). These studies suggest that *H. indica* has comparable heat tolerance to Hb, while *H. megditis* has lower heat tolerance than Hb, similar heat tolerance as Sf, and similar infection levels as Sc when at optimal, mild temperatures.

In a study by Grewal et al. (1994), *S. riobrave* was the only species to infect *G. mellonella* at 39°C, and it infected at about twice the rate at 37°C compared to either strain of *S. glaseri*, which was the only other species to infect at 37°C. This study also included Hb, Sc, and Sf, but they did not infect above 32 or 35°C. Grewal et al. (1994) also reported that *S. glaseri* caused approx. 60 to 70% infection at 10°C, which was a level of infection lower only than caused by Sf at that temperature. In a study by Kung et al. (1991), *S. glaseri* survival was statistically no different at 5 or 35°C until after 16 weeks exposure, when survival was slightly lower at 5°C. Molyneux (1985) reported that *S. glaseri* had higher survival than Sc at temperatures 15 to 28°C at any exposure up to 32 weeks, and higher survival at 10°C as well, for exposures longer than 16 weeks. Infectivity of *S. glaseri* against *L. cuprina* was higher than infectivity of Sc for temperature 10 to 28°C at any exposure up to 32 weeks. These studies indicate that *S. riobrave* is the most heat tolerant of EPN species, while *S. glaseri* has a relatively wide range for infection, at both temperature extremes.

Conclusions

There is a wide range of effects of temperature on the three most commercially available species of EPNs, Hb, Sc, and Sf, with influence from both strain and duration of exposure. Most studies suggest that Hb and Sc have similar, high tolerance to heat, though some studies with varied strains report one species or the other as more tolerant. Most studies describe Sf is as having a lower range for survival and infectivity, with lower cold and hot temperature limits. Many other species of *Heterorhabditis* and *Steinernema* have been examined in isolated studies, but they are less often used as biological control agents, so these studies are largely academic.

When considering which EPN species to apply for pest management in agricultural or horticultural settings, growers must choose a species that will survive and remain infective in the existing growing conditions. It is true that high temperature tolerance is only one factor determining success of pest control by EPNs, and that most research has not been conducted with commercial EPN

strains. However, comparing existing research on EPN high temperature tolerance to actual growing conditions could still help identify the best species to apply, reducing the probability of a lapse in pest control as temperatures increase.

Table 1.1. Summary of research on the effects of temperature on EPNs, listed in the order discussed in the text. Strains were wild isolated (W), lab cultures (L), or commercially produced (C). These studies included a focus on the effect of temperature on EPN survival (S), infectivity (I), or both.

Reference	Species*	Source	Temperature (°C)	Durations	Focus	Host
Ehlers et al. 2005	Hb	L: inbred lines	37.6 – 39.8	n/a	S	n/a
Mukuka et al. 2010	Hb, Hi, Hm	W: 36 isolates L: 17 hybrids C: one hybrid	32 – 41	3h pre. 2h treat.	S	n/a
Susurluk and Ulu 2015	Hb, Sc, Sf	W: Turkey	38	2h	S	n/a
Morton and García-del-Pino 2009	Hb, Sc, Sf	W: Mediterranean	S: 25 – 42 I: 5 – 37	S: 2- 12h I: every 24h	S, I	*Galleria mellonella*
Finnegan et al. 1999	Hb	W: Hungary	S: 32 – 39 I: 33 – 39	1h	S, I	*G. mellonella*
Chung et al. 2010	Hb	W: Korea	13 – 35	Every 24h	I	*G. mellonella*
Grewal et al. 1994	Hb, Sc, Sf, Hm, Sg, Sr, Ss	L: Hb, Sc- USA; Sf- Argentina, France	I: 8 – 39	Every 8h, up to 20d	I	*G. mellonella*
Molyneux 1986	Hb	W: New Zealand	0 – 40	10-28d	I	*Lucilia cuprina*
Mahar et al. 2005	Hb, Sc, Sf, Hi	L: Hb- HW79, Sc- "All", W: Sf- UK	20 or 25	2d	I	*Otiorhynchus sulcatus*
Shapiro et al. 1999	Hb, Hi	L: Hb1 strain	21 – 27	14d	I	*Diaprepes abbreviatus*
Kung et al. 1991	Sc	C: BioSys, Inc.	5 – 35	Up to 96d	S, I	*G. mellonella*
Somasekhar et al. 2002	Sc	W: Eastern USA C: one strain	40	2h	S, I	*G. mellonella*
Ali et al. 2007	Sc	W: India	15 – 45	6h	S, I	*Helicoverpa armigera*
Grewal et al. 1993	Sc	L: "All" strain	15 – 37.5	Every 24h for 4d	I	*G. mellonella*
Grewal et al. 1999	Sc	L: produced in vivo and in vitro	28	48h	I	*G. mellonella*
Saunders and Webster 1999	Sc, Hm	L: "All" strain	8 – 24	4x/day, for 4d	I	*G. mellonella*
Radova and Trnkova 2010	Sc, Sf	L	10 – 25	5, 7, 14d	I	*Tenebrio molitor*
Addis et al. 2011	Sf	W: Eastern Java, Indonesia	S: 37 – 41	2h	S	n/a
Molyneux 1985	Sf, Sg	L: "Agriotos" strain	10 – 28	Up to 32w	S, I	*G. mellonella* *L. cuprina*
Menti et al. 2000	Sf, Hm	W: Sf: Greece, UK	I: 10 – 35	3d	I	*G. mellonella*

*Species are abbreviated as follows: *H. bacteriophora* (Hb), *S. carpocapsae* (Sc), *S. feltiae* (Sf), *H. indica* (Hi), *H. megditis* (Hm), *S. glaseri* (Sg), *S. riobrave* (syn. *S. riobravis*) (Sr), and *S. scaptericsi* (Ss).

REFERENCES

Abbott, W. S. 1925. A Method of Computing the Effectiveness of an Insecticide. J. Econ. Entomol. 3: 302–303.

Abu Hatab, M. A., and R. Gaugler. **1997**. Influence of growth temperature on fatty acids and phospholipids of Steinernema riobravis infective juveniles. J. therm. Biol. 22: 237–244.

Addis, T., M. Mulawarman, L. Waeyenberge, M. Moens, N. Viaene, and R. U. Ehlers. **2011**. Identification and intraspecific variability of Steinernema feltiae strains from Cemoro Lawang village in Eastern Java, Indonesia. Russ. J. Nematol. 19: 21–29.

Ali, S. S., R. Pervez, M. Abid Hussain, and R. Ahmad. **2007**. Effect of temperature on survival of Steinernema seemae, S. masoodi and S. carpocapsae (Rhabditida: Steinernematidae) and their subsequent infectivity to prepupa of Helicoverpa armigera (Hübner). Arch. Phytopathol. Plant Prot. 40: 183–187.

Andaló, V., A. Moino, C. Maximiniano, V. P. Campos, and L. A. Mendonça. **2011**. Influence of temperature and duration of storage on the lipid reserves of entomopathogenic nematodes. Rev. Colomb. Entomol. 37: 203–209.

Ansari, M. A., and T. M. Butt. **2011**. Effect of potting media on the efficacy and dispersal of entomopathogenic nematodes for the control of black vine weevil, Otiorhynchus sulcatus (Coleoptera: Curculionidae). Biol. Control. 58: 310–318.

Bateman, R. P., G. A. Matthew, and F. R. Hall. **2007**. Ground-based application technology. *In* F. Man. Tech. Invertebr. Pathol.

van den Bosch, R. 1982. An introduction to biological control, 2nd ed. Springer Science+Business Media, New York, NY.

Braun, S. E., J. P. Sanderson, E. B. Nelson, M. L. Daughtrey, and S. P. Wraight. **2009**. Fungus gnat feeding and mechanical wounding inhibit Pythium aphanidermatum infection of geranium seedlings. Phytopathology. 99: 1421–1428.

Buitenhuis, R., and J. L. Shipp. **2005**. Efficacy of entomopathogenic nematode Steinernema feltiae (Rhabditida: Steinernematidae) as influenced by Frankliniella occidentalis (Thysanoptera: Thripidae) developmental stage and host plant stage. J. Econ. Entomol. 98: 1480–1485.

Campbell, L. R., and R. Gaugler. **1991**. Mechanisms for exsheathment of entomopathogenic nematodes. Int. J. Parasitol. 21: 219–224.

Chung, H. J., D. W. Lee, H. S. Yoon, S. M. Lee, C. G. Park, and H. Y. Choo. **2010**. Temperature and dose effects on the pathogenicity and reproduction of two Korean isolates of Heterorhabditis bacteriophora (Nematoda: Heterorhabditidae). J. Asia. Pac. Entomol. 13: 277–282.

Ciche, T. A., and J. C. Ensign. **2003**. For the insect pathogen Photorhabdus luminescens, which end of a nematode is out? Appl. Environ. Microbiol. 69: 1890–1897.

Croll, N. A., and B. E. Matthews. **1973**. Activity, ageing and penetration of hookworm larvae. Parasitology. 66: 279–289.

Cuce, E., D. Harjunowibowo, and P. M. Cuce. **2016**. Renewable and sustainable energy saving strategies for greenhouse systems: A comprehensive review. Renew. Sustain. Energy Rev. 64: 34–59.

Cuthbertson, A. G. S., K. F. A. Walters, P. Northing, and W. Luo. **2007**. Efficacy of the entomopathogenic nematode, Steinernema feltiae, against sweetpotato whitefly Bemisia tabaci (Homoptera: Aleyrodidae) under laboratory and glasshouse conditions. Bull. Entomol. Res. 97: 9.

Dowd, B., and A. Peters. **2002**. Virulence mechanisms, pp. 79–98. *In* Gaugler, R. (ed.), Entomopathog. Nematol. CABI Publishing, New York, NY.

Ehlers, R. U., J. Oestergaard, S. Hollmer, M. Wingen, and O. Strauch. **2005**. Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode-bacterium complex Heterorhabditis bacteriophora- Photorhabdus luminescens. BioControl. 50: 699–716.

Fife, J. P., H. E. Ozkan, R. C. Derksen, and P. S. Grewal. **2007**. Effects of pumping on entomopathogenic nematodes and temperature increase within a spray system. 23: 405–412.

Finnegan, M. M., M. J. Downes, M. O'Regan, and C. T. Griffin. **1999**. Effect of salt and temperature stresses on survival and infectivity of Heterorhabditis spp. IJs. Nematology. 1: 69–78.

Fitters, P. F. L., and C. T. Griffin. **2004**. Spontaneous and induced activity of Heterorhabditis megidis infective juveniles during storage. Nematology. 6: 911–917.

Fitters, P. F. L., and C. T. Griffin. **2006**. Survival, starvation, and activity in Heterorhabditis megidis (Nematoda: Heterorhabditidae). Biol. Control. 37: 82–88.

Gang, S. S., and E. A. Hallem. **2016**. Mechanisms of host seeking by parasitic nematodes. Mol. Biochem. Parasitol. 208: 23–32.

Georgis, R., A. M. Koppenhöfer, L. A. Lacey, G. Bélair, L. W. Duncan, P. S. Grewal, M. Samish, L. Tan, P. Torr, and R. W. H. M. van Tol. **2006**. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control. 38: 103–123.

Grewal, P. S. 2000. Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida: Steinernematidae). Int. J. Parasitol. 30: 995–1000.

Grewal, P. S., V. Converse, and R. Georgis. **1999**. Influence of production and bioassay methods on infectivity of two ambush foragers (Nematoda: Steinernematidae). J. Invertebr. Pathol. 73: 40–44.

Grewal, P. S., R. U. Ehlers, and D. I. Shapiro-ilan. **2005**. Nematodes as Biocontrol Agents.

Grewal, P. S., R. Gaugler, H. K. Kaya, and M. Wusaty. **1993**. Infectivity of the entomopathogenic nematode Steinernema scapterisci (Nematoda: Steinernematidae). J. Invertebr. Pathol.

Grewal, P. S., S. Selvan, and R. Gaugler. **1994**. Thermal adaptation of entomopathogenic nematodes:

niche breadth for infection, establishment, and reproduction. J. Therm. Biol. 19: 245–253.

Gumus, A., M. Karagoz, D. Shapiro-Ilan, and S. Hazir. **2015**. A novel approach to biocontrol: release of live insect hosts pre-infected with entomopathogenic nematodes. J. Invertebr. Pathol. 130: 56–60.

Van Gundy, S. D., A. F. Bird, and H. R. Wallace. **1967**. Aging and starvation in larvae of Meloidogyne javanica and Tylenchulus semipenetran. Phytopathology. 57: 559–57.

Hashmi, G., S. Hashmi, S. Selvan, P. Grewal, and R. Gaugler. **1997**. Polymorphism in heat shock protein gene (hsp70) in entomopathogenic nematodes (Rhabditida). J. Therm. Biol. 22: 143–149.

Hashmi, S., G. Hashmi, I. Glazer, and R. Gaugler. **1998**. Thermal response of Heterorhabditis bacteriophora transformed with the Caenorhabditis elegans hsp70 encoding gene. J. Exp. Zool. 281: 164–170.

Hass, B., M. Downes, and C. T. Griffin. **2002**. Persistence of four Heterorhabditis spp. isolates in soil: role of lipid reserves. J. Nematol. 34: 151–158.

Helmberger, M. S., E. J. Shields, and K. G. Wickings. **2017**. Ecology of belowground biological control: Entomopathogenic nematode interactions with soil biota. Appl. Soil Ecol. 121: 201–213.

Hiltpold, I. 2015. Prospects in the application technology and formulation of entomopathogenic nematodes for biological control of insect pests, pp. 187–205. *In* Campos-Herrera, R. (ed.), Nematode Pathog. Insects Other Pests. Springer, New York.

Hominick, W. M., B. R. Briscoe, F. G. del Pino, J. Heng, D. J. Hunt, E. Kozodoy, Z. Mracek, K. B. Nguyen, a. P. Reid, S. Spiridonov, P. Stock, D. Sturhan, C. Waturu, and M. Yoshida. **1997**. Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. J. Helminthol. 71: 271.

Jagdale, G. B., M. L. Casey, P. S. Grewal, and R. K. Lindquist. **2004**. Application rate and timing, potting medium, and host plant effects on the efficacy of Steinernema feltiae against the fungus gnat, Bradysia coprophila, in floriculture. 29: 296–305.

Jagdale, G. B., and R. Gordon. **1997**. Effect of temperature on the composition of fatty acids in total lipids and phospholipids of entomopathogenic nematodes. J. Therm. Biol. 22: 245–251.

Jagdale, G. B., P. S. Grewal, and S. O. Salminen. **2005**. Both heat-shock and cold-shock influence trehalose metabolism in an entomopathogenic nematode. J. Parasitol. 91: 988–994.

Johnigk, H. A., S. Hollmer, O. Strauch, U. Wyss, and R. U. Ehlers. **2002**. Heritability of the liquid culture mass production potential of the entomopathogenic nematode Heterorhabditis bacteriophora. Biocontrol Sci Technol. 12: 267–276.

Kaspi, R., A. Ross, A. K. Hodson, G. N. Stevens, H. K. Kaya, and E. E. Lewis. **2010**. Foraging efficacy of the entomopathogenic nematode Steinernema riobrave in different soil types from California citrus groves. Appl. Soil Ecol. 45: 243–253.

Kaya, H. K., M. M. Aguillera, A. Alumai, H. Y. Choo, M. de la Torre, A. Fodor, S. Ganguly, S. Hazir, T. Lakatos, A. Pye, M. Wilson, S. Yamanaka, H. Yang, and R. U. Ehlers. **2006**. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol. Control. 38: 134–155.

Kaya, H. K., and R. Gaugler. **1993**. Entomopathogenic nematodes. Annu. Rev. Entomol. 38: 181–206.

Kung, S.-P., R. Gaugler, and H. K. Kaya. **1991**. Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. J. Invertebr. Pathol. 57: 242–249.

Leite, L. G., D. I. Shapiro-Ilan, and S. Hazir. **2018**. Survival of Steinernema feltiae in different formulation substrates: Improved longevity in a mixture of gel and vermiculite. Biol. Control. 126: 192–197.

Lewis, E. E., J. Campbell, C. Gri, H. Kaya, and A. Peters. **2006**. Behavioral ecology of entomopathogenic nematodes. Biol. Control. 38: 66–79.

Lewis, E. E., and D. J. Clarke. **2012**. Nematode parasites and entomopathogens, Second Edi. ed, Insect Pathol. Elsevier.

Mahar, A. N., D. A. Darban, A. G. Lanjar, M. Munir, N. D. Jan, N. G. M. Hague, and S. R. Gowen. **2005**. Influence of temperature on the production and infectivity of entomopathogenic nematodes against larvae and pupae of vine weevil, Otiorhynchus sulcatus (Coleoptera: Curculionidae). J. Entomol.

Menti, H., D. J. Wright, and R. N. Perry. **2000**. Infectivity of populations of the entomopathogenic nematodes Steinernema feltiae and Heterorhabditis megidis in relation to temperature, age and lipid content. Nematology. 2: 515–521.

Molyneux, A. S. 1985. Survival of infective juveniles of Heterorhabditis spp., and Steinernema spp. (Nematoda : Rhabditida) at various temperatures and their subsequent infectivity for insects. Rev. Nématologie. 8: 165–170.

Molyneux, A. S. 1986. Heterorhabditis spp. and Steinernema (= Neoaplectana) spp.: temperature, and aspects of behavior and infectivity. Exp. Parasitol. 62: 169–180.

Morton, A., and F. García-del-Pino. **2009**. Ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean areas. J. Invertebr. Pathol. 102: 203–213.

Mukuka, J., O. Strauch, L. Waeyenberge, N. Viaene, M. Moens, and R. U. Ehlers. **2010**. Heat tolerance among different strains of the entomopathogenic nematode Heterorhabditis bacteriophora. BioControl. 55: 423–434.

Patel, M. N., M. Stolinski, and D. J. Wright. **1997**. Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four Steinernema species. Parasitology. 114: 489–496.

Patel, M. N., and D. J. Wright. **1997**. Glycogen: its importance in the infectivity of aged juveniles of Steinernema carpocapsae. Parasitology. 114: 591–596.

Peters, A., R. Han, X. Yan, and L. G. Leite. **2016**. Production of entomopathogenic nematodes, pp. 157–170. *In* Microb. Control Insect Mite Pests from Theory to Pract. Elsevier Inc.

Poinar, G. O., and P. S. Grewal. **2012**. History of entomopathogenic nematology. J. Nematol. 44: 153–61.

Radová, Š., and Z. Trnková. **2010**. Effect of soil temperature and moisture on the pathogenicity of two species of entomopathogenic nematodes (Rhabditida: Steinernematidae). J. Agrobiol. 27: 1–7.

San-Blas, E. 2013. Progress on entomopathogenic nematology research: a bibliometric study of the last three decades: 1980-2010. Biol. Control. 66: 102–124.

Saunders, J. E., and J. M. Webster. **1999**. Temperature effects on Heterorhabditis megidis and Steinernema carpocapsae infectivity to Galleria mellonella. J. Nematol. 31: 299–304.

Selvan, S., R. Gaugler, and E. E. Lewis. **1993**. Biochemical energy reserves of entomopathogenic nematodes. J. Parasitol. 79: 167–172.

Shapiro-Ilan, D. I., S. Hazir, and L. Lete. **2015**. Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. J. Nematol. 47: 184–189.

Shapiro-Ilan, D. I., E. E. Lewis, and P. Schliekelman. **2014**. Aggregative group behavior in insect parasitic nematode dispersal. Int. J. Parasitol. 44: 49–54.

Shapiro, D. I., J. R. Cate, J. Pena, A. Hunsberger, and C. W. Mccoy. **1999**. Effects of temperature and host age on suppression of Diaprepes abbreviatus (Coleoptera: Curculionidae) by entomopathogenic nematodes. J. Econ. Entomol. 92: 1086–1092.

Smits, P. H. 1996. Post-application persistence of entomopathogenic nematodes. Biocontrol Sci. Technol. 6: 379–387.

Snyder, H., S. P. Stock, S. K. Kim, Y. Flores-Lara, and S. Forst. **2007**. New insights into the colonization and release processes of Xenorhabdus nematophila and the morphology and ultrastructure of the bacterial receptacle of its nematode host, Steinernema carpocapsae. Appl. Environ. Microbiol. 73: 5338–5346.

Solomon, A., R. Salomon, I. Paperna, and I. Glazer. **2000**. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. Parasitology. 121: 409–416.

Somasekhar, N., P. S. Grewal, and M. G. Klein. **2002**. Genetic variability in stress tolerance and fitness among natural populations of Steinernema carpocapsae. Biol. Control. 310: 303–310.

Susurluk, I. A., and T. C. Ulu. **2015**. Virulence comparisons of high-temperature-adapted Heterorhabditis bacteriophora, Steinernema feltiae and S. carpocapsae. Helminthol. 52: 118–122.

CHAPTER 2

THE IMPACT OF HIGH TEMPERATURE EXPOSURE ON THE SURVIVAL OF COMMERCIALLY AVAILABLE ENTOMOPATHOGENIC NEMATODES

Introduction

Entomopathogenic nematodes (EPNs) are used widely in agriculture and horticulture as a biological control agent, mostly against soil-dwelling insect pests (Georgis et al. 2006). They are mass reared by companies that use highly controlled in vitro techniques, then infective juveniles (IJs) are shipped in a quiescent state in various product formulations to maintain viability (Grewal 2000, Peters et al. 2016). Currently, most commercial production of EPNs is focused on *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) (Hb), *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) (Sc), and *Steinernema feltiae* Filipjev (Sf), although several other species are produced for specific markets (Kaya et al. 2006).

To ensure successful pest control by EPNs, many potential limiting factors must be considered. Even when applied as inundative releases without expectation of long-term persistence, EPNs must survive long enough to be effective after each application (Smits 1996). Abiotic factors that can affect the success of EPNs in pest control include soil type, moisture, UV radiation, and, importantly, temperature (Jagdale et al. 2004, Kaspi et al. 2010, Shapiro-Ilan et al. 2015). Climate control within greenhouses can sometimes be inefficient and expensive (Cuce et al. 2016), so many businesses must cope with suboptimal production temperatures at certain times of year. This series of studies sought to evaluate the effects of high temperature, duration of exposure, and product formulation on the survival of the three most common commercially available EPN species.

Many previous studies have examined the effects of temperature on EPN survival, each focusing on a subset of species, strains, temperature ranges, and durations of exposure. Although a few studies have included commercial strains of EPNs, most research has focused on wild isolates. Overall, these

studies suggest that Hb and Sc have similar, high tolerance to heat, though sometimes one species is reported as significantly more tolerant than the other, likely due to strain differences. The temperature range for survival of Sf is frequently reported to be the lowest of the three species.

Only two studies have examined the effects of high temperatures on the survival of Hb, Sc, and Sf concurrently, using wild strains isolated from around the world. Susurluk and Ulu (2015) calculated the mean temperature to cause 50% mortality (LT_{50}) for each species, and reported Hb as the most tolerant, followed closely by Sf, then Sc. The LT_{50} values only ranged from 35.5 to 38.1°C, overall. Morton and García-del-Pino (2009) observed Hb to be most heat tolerant, but Sf as least tolerant. After treatment for 4 h at 32°C, about 55% of Hb and Sc survived, while most strains of Sf had about 45% survival, with survival of some strains as low as 10%.

Other research has examined the survival of just one or two of these EPN species. Mukuka et al. (2010) reported the LT_{50} for strains of Hb to range from 33.3 to 40.1°C, indicating that tolerance can vary widely among strains of the same species. Also, some of these LT_{50} values are lower than reported for Sc and Sf by Susuluk and Ulu (2015), suggesting that EPN species can overlap in tolerance, when accounting for strain variation. Several other studies have focused on Sc, reporting it can have very high heat tolerance. After treating eleven isolates of Sc from the eastern USA for 2 h at 40°C, Somasekhar et al. (2002) reported 37 to 82% EPN survival, with nine of the isolates maintaining survival above 60%. Ali et al. (2007) examined Sc isolated from India, and after 6 h treatment at 45°C they observed only 50% EPN mortality.

Formulation of EPN products may also affect pest control at different temperatures. Commercial EPNs are kept in a quiescent, anhydrobiotic state through shipment, usually in a polyacrylamide gel or powder-based matrix, though exact components of these formulations are usually not disclosed. A study by Leite et al. (2018) tested EPN carriers and formulations such as gel, diatomaceous earth, potting mix, compost, among others. The survival at 15°C of an isolate of Sf from France was highest in gel or water,

at 25°C it was highest in gel or vermiculite, and at 35°C it was highest in peat. This suggests that gel-based formulations provide robust pest control in moderate climates, however, commercial strains EPNS were not included in the study, and neither was a powder-based formulation.

Our aim in this study was to examine the effect of high temperatures, duration of high temperature exposure, and product formulation on the survival of commercially available strains of Hb, Sc, and Sf. The first experiment examined the effects of duration of exposure at various temperatures, then similar experiments were conducted in a way that comparisons could be made between species and formulations as well. These studies will provide insight into the environmental limits of EPNs currently being used in agriculture and horticulture, and better characterize their control of an important greenhouse pest. The results will enable those making pest management decisions to have greater certainty when choosing and applying EPNs.

Materials and Methods

Source of EPNs

Three commonly used species of EPNs, *Heterorhabditis bacteriophora* (Hb), *Steinernema carpocapsae* (Sc), and *Steinernema feltiae* (Sf), were each provided by three biocontrol companies: BASF, E-nema, and Koppert Biological Systems, which were treated as three separate strains for each species. These companies use liquid culture for mass rearing of EPNs, and are the suppliers for most EPN distributors around the world. The companies vary in EPN product formulation; BASF and Koppert Biological Systems use gel-based preservation, while E-nema usually preserves EPNs in a powder-based formulation, although Sf can be formulated in either. Products were sent with expedited shipping, and specialized packaging was used to preserve them at a cool temperature. Upon receipt of each shipment, products were placed in a refrigerator at 5°C for storage until use. The effect of high temperatures on EPN survival was tested with the following three experiments.

Effect of Duration of High Temperature Exposure on EPN Survival

The effect of duration of high temperature exposure on EPN survival was tested with a two-day process. On the first day, a small sample of each commercial strain of one EPN species was removed from the stored packages and allowed to warm up to room temperature. The most common product formulation from each company was used: gel-based products from BASF and Koppert Biological Systems, and powder-based products from E-nema. EPNs were then added to 25°C deionized water in 30 mL glass test tubes and diluted to approximately 2000 IJs/mL. Then, 0.25 mL of this solution was added to 0.75 mL water in glass vials (Kimble: 15x45 mm 1 Dram), either at 25°C for controls, or preheated to the treatment temperature of that setup day, resulting in 1 mL with approximately 500 IJs in each vial. These vials were kept in trays, and a separate tray was set up for each exposure duration (0, 1, 2, 4, 6, 8, and 10 h), each containing eight vials for each commercial strain.

The tray with control vials, which would receive 0 h treatment at high temperature, was placed directly into the 25°C incubator. The rest of the trays were placed in an incubator set to the single high temperature being tested that day, 30, 35, 40, or 45°C, then transferred to the 25°C incubator after the allotted treatment duration. Temperature was monitored in the incubators using a digital data logger (Onset: HOBO UX100-003). Temperatures remained within 1°C of the target treatment temperature throughout each treatment period. Adding 0.25 mL EPN solution to preheated water in the vials helped to minimize the time it took to warm up to the target treatment temperature. Vials were provided an additional warm-up period in the treatment incubator (10, 15, 20, and 25 min for 30, 35, 40, and 45°C, respectively) in order to reach the target temperature before the timing for duration of exposure began. All trays remained in the 25°C incubator overnight, to allow EPNs time for recovery from heat shock, if needed, before mortality was assessed (Somasekhar et al. 2002).

The next day, one sample (0.05 mL) was taken from each vial, and mortality of EPNs was assessed. To take a sample, vials were lightly agitated until EPNs were evenly distributed by visual

evaluation, then 0.05 mL was extracted with a pipette from the center of the water column. Drops were placed on a petri dish, examined under a dissecting microscope, and EPNs were counted as alive if they were actively moving, or if they moved after being gently rolled with a fine, flexible hair probe. To examine EPN mortality due to high temperature, the mortality in each sample was corrected based on that day's average control mortality for the EPN species and strain, according to Abbott's formula (Abbott 1925):

$$\text{Corrected proportion mortality} = \frac{\text{Mortality in sample} - \text{Average control mortality}}{1 - \text{Average control mortality}}$$

This experiment was repeated on one setup day for each combination of EPN species and treatment temperature, using a single production batch from each company.

Effects of Species and Duration of High Temperature Exposure on EPN Survival

To test the combined effects of EPN species and duration of high temperature exposure on the survival of EPNs, the same process as above was used, except with the following modifications: All three species were tested on each day, with three replicate vials for each combination of species, strain, and duration of exposure. This was repeated on three setup days for each of the treatment temperatures, 30, 35, 40, and 45°C. Then, the complete experiment was conducted a total of three times, with distinct production batches from the companies.

Effect of Product Formulation on EPN Survival at High Temperatures

To test the effect of product formulation on the survival of EPNs at high temperatures, the gel formulation of Sf from E-nema was also treated during the previous experiment, with the same number of replicates, and compared to the powder formulation of Sf from E-nema, the same EPN, in a separate analysis.

Statistical Analysis

For the experiment testing only the effect of duration of exposure on EPN survival, a linear mixed effects model was fitted for each combination of species and temperature. The response variable

was the Abbott's corrected proportion of EPN mortality. Each model included the fixed effect of duration, and the random effect of EPN strain. No statistical comparisons between EPN species was made, since they were not treated concurrently.

To compare the effects of EPN species and duration of exposure on EPN survival, a linear mixed effects model was fitted, using data from the most common product formulation of each commercial strain. The response variable was the Abbott's corrected proportion of EPN mortality. The model included fixed effects of species, temperature, and duration, and their two and three-way interactions.

To check the effect of product formulation on the survival of Sf at high temperatures, a linear mixed effects model was fitted, using Abbott's corrected proportion of Sf mortality as the response variable. The model included fixed effects of temperature, duration, formulation, and their two and three-way interactions, and random effects of EPN batch and setup day.

Posthoc tests were performed for each model, using Tukey's HSD to control for multiple comparisons. A residual analysis was performed for all models, to check the model assumptions of normality and homogeneity. Analyses were performed with R studio (version 3.4.3).

Variability of data means is reported with the standard error.

Results

Effect of Duration of High Temperature Exposure on EPN Survival

Mortality of EPNs after various treatments is shown in Figure 2.1. Treatment duration had no significant effect at 30°C, but it did have an effect at 35 and 40°C (Table 2.1). All treatment durations at 45°C resulted in mean mortality above 99% for all three EPN species (data not shown).

At 35°C, Sc mortality only varied significantly between 1 and 8 h treatments ($p=0.021$), although this only represents a 4% increase in mean mortality (Fig. 2.1E). Mortality of Sf remained stable until a significant increase between the 4 to 6 h , 6 to 8 h, and 8 to 10 h treatments ($p<0.001$, $p<0.001$, $p<0.001$;

Fig 2.1H). The largest increase in Sf mean mortality was from 7 ± 1.5% after 4 h treatment to 54 ± 5.8% after 6 h treatment. The only consecutive duration treatments that resulted in a significant increase in Hb mortality were 2 and 4 h, although there was also a significant increase between 4 and 8 h (p=0.001, p<0.001; Fig. 2.1B). Mortality of Sc, Hb, and Sf varied greatly after 8 h treatment at 35°C, with mean mortalities of 2.8 ± 1.2, 47.1 ± 4.6, and 80.8 ± 5.2%, respectively.

At 40°C, the mean mortality of Sc remained below 5% after 1 and 2 h treatments, with no significant increase, but there was a large increase from 2 to 4 h, 4 to 6 h, and 6 to 8 h, the latter ending with 100% mortality (p=0.664, p<0.001, p<0.001, p=0.011; Fig. 2.1F). After just 1 h treatment at 40°C, Sf and Hb had mean mortalities of 36 ± 3.0 and 32 ± 3.3%. Mortality of Sf and Hb increased significantly between 1 and 2 h, resulting in 100% and 87 ± 3.2%, respectively, then mortality of Hb increased further between 2 h and 4 h, resulting in 100% mortality (p<0.001, for all comparisons; Fig. 2.1C,I).

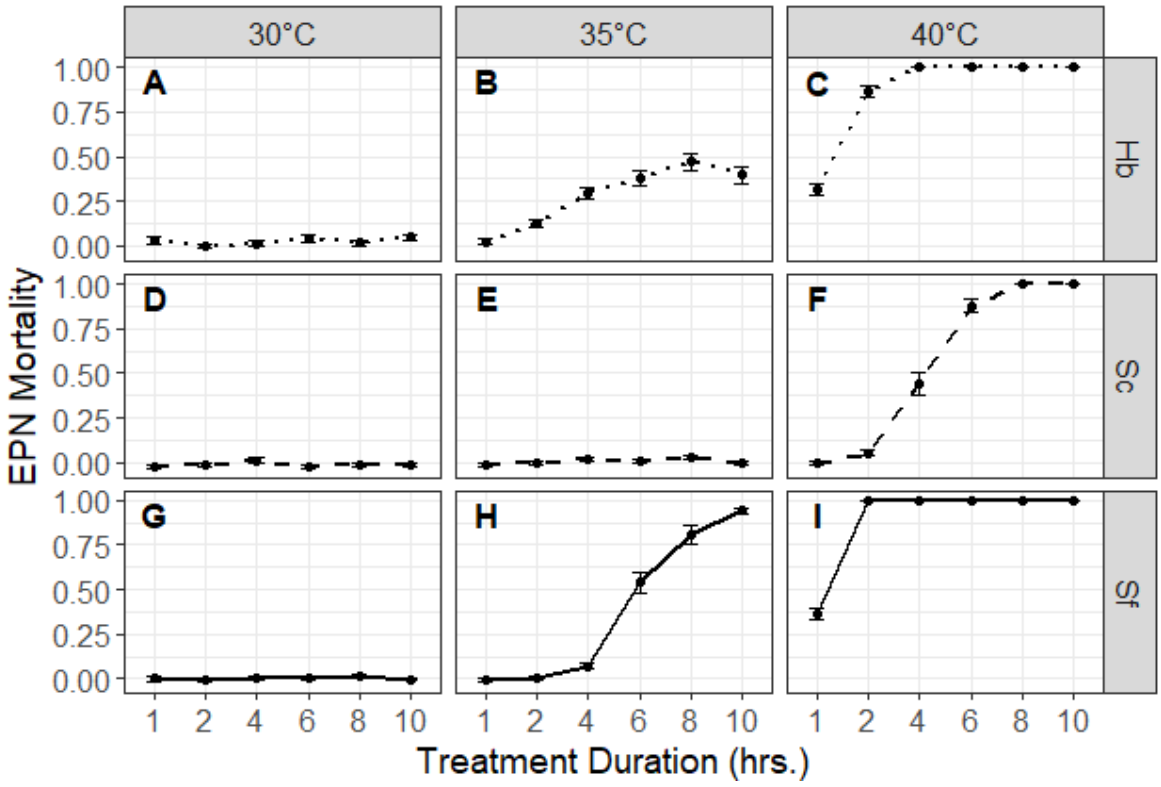

Figure 2.1. Mean proportion of mortality of three EPN species, corrected with Abbott's formula, after treatment at various high temperatures and durations of exposure. Error bars represent +/- one standard error.

Table 2.1. ANOVA results for 9 separate models, each analyzing treatments of one species at one temperature. Degrees of freedom are 5 for the numerator and 136 for the denominator for each of these models. Significance of treatment duration within each model is shown.

Species	Temp (°C)	F value	p (* = significant)
Hb	30	1.5565	0.1766
Sc	30	2.0769	0.07201
Sf	30	0.9943	0.4237
Hb	35	36.189	<0.0001 *
Sc	35	2.7025	0.0231 *
Sf	35	255.57	<0.0001 *
Hb	40	228.5	<0.0001 *
Sc	40	334.62	<0.0001 *
Sf	40	463.07	<0.0001 *

Effects of Species and Duration of High Temperature Exposure on EPN Survival

There was a wide range in the proportion mortality of heat-treated EPNs, with a significant effect of the three-way interaction between species, temperature, and duration (Fig. 2.2; $F_{30, 5076}$=83.73, p<0.001).

There was no significant difference between the mortality at 30°C for any combination of EPN species and duration (p>0.05). There was no difference between species at 35°C until 4 h duration, when Hb mortality was significantly greater than that of Sc or Sf (p<0.001, p=0.002, respectively). Only Sc had statistically lower mortality at 6 h, then the species had statistically increasing mortalities (Sc<Hb<Sf) after both 8 and 10 h duration treatments (p<0.001, for all comparisons). After 10 h treatment at 35°C, the mean mortalities for Sc, Hb, and Sf were 5.3 ± 1.0, 40.6 ± 3.3, and 70.3 ± 3.5%, respectively.

The same order of statistically increasing mortalities of species (Sc<Hb<Sf) was observed after 1 and 2h treatment at 40°C (p<0.001). Mean mortality surpassed 80% at 2 h exposure for Sf, at 4 h for Hb, and not until 8 h for Sc. Mortality of Sc remained lower than Sf or Hb through 8 h exposure at 40°C, with a mean mortality of only 17.6 ± 3.0, 35.1 ± 3.3, and 66.4 ± 3.6% after 2, 4, and 6 h treatments (p<0.001, for all comparisons). Mortality of Sc was no different from Hb after 10 h (p=0.564).

At 45°C, Sc had statistically lower mean mortality, 25.9 ± 2.3%, after 1 h exposure ($p<0.001$), but for all longer durations mortality was 100% for all species.

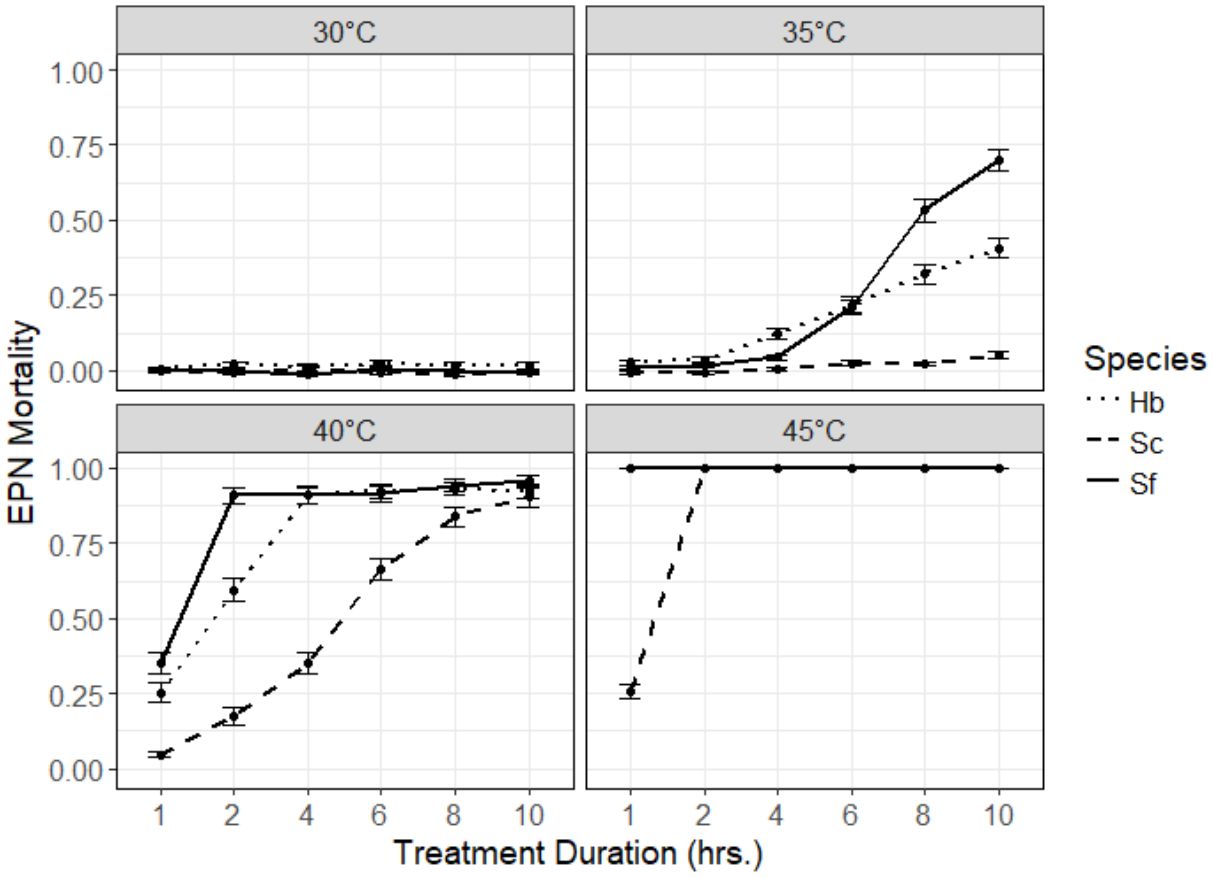

Figure 2.2. Mean proportion of EPN mortality, corrected with Abbott's formula, after treatment at various high temperatures and for various durations. Error bars represent +/- one standard error.

Effect of Product Formulation on EPN Survival at High Temperatures

The mortality of Sf with different product formulations after treatment at high temperatures is shown in Figure 2.3. There was a significant three-way interaction between temperature, duration, and formulation ($F_{10, 929}=2.034$, $p=0.027$). However, formulation only had a significant effect after 10 h at 35°C, when Sf in powder formulation had higher mortality, and after 1 h at 40°C, when Sf in gel formulation had higher mortality ($p<0.001$, $p=0.004$).

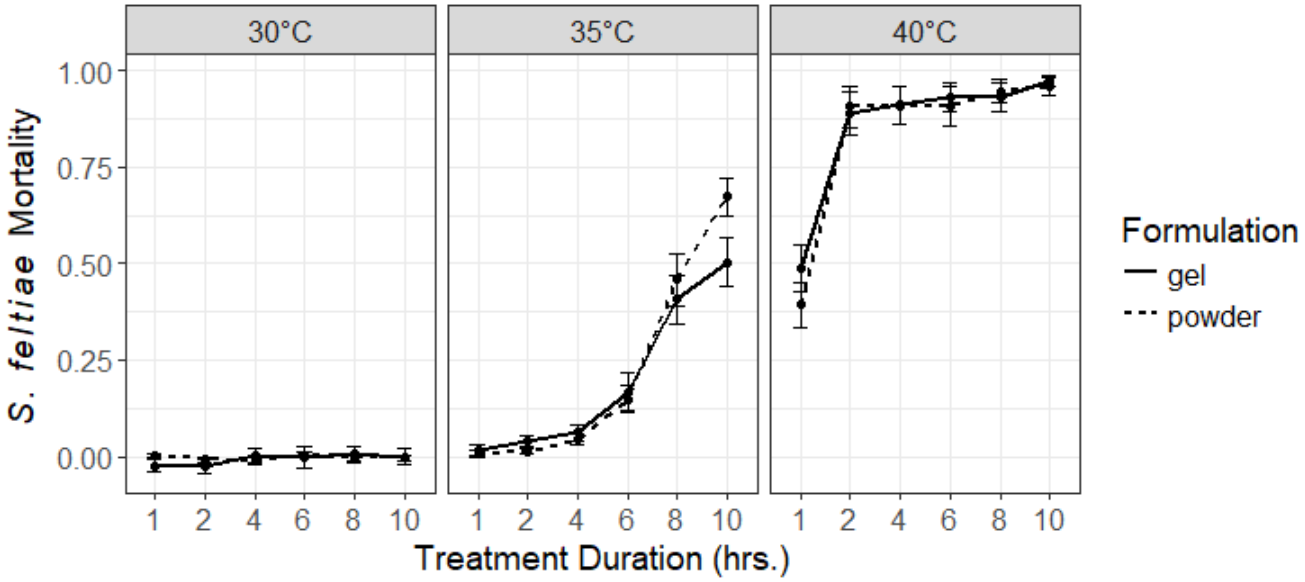

Figure 2.3. Mean proportion of Sf mortality, corrected with Abbott's formula. A single commercial strain was tested, in both gel and powder formulations. Error bars represent +/- one standard error.

Discussion

Species, temperature, product formulation, and duration of high temperature exposure can all have an impact on EPN survival. In the first experiment of this study, duration of exposure was shown to have a significant effect for various EPN species at some temperatures. Duration of exposure had no effect on any species at 30°C, though it did affect Hb and Sf at 35°C, starting at 4 and 6 h treatments, respectively. Longer durations of exposure at 35°C did not continue to affect Hb significantly. Duration of exposure had a significant effect on the survival of all three EPN species at both 40°C, while at 45°C it only impacted Sc survival, as the other two species had complete mortality after all durations of exposure.

Further testing revealed that EPN species can differ from each other in survival at high temperatures, when considering duration of exposure as well. Treatments at 30°C had no impact on the mortality of any EPN species, and the species did not differ from each other. Treatments at 35°C, however, revealed a clear difference in heat tolerance. There was higher mortality of Hb than of other

species after 4 h treatment at 35°C, but then Sf mortality was highest after 8 and 10 h treatments. Mortality of Sc was lower than both Hb and Sf after 6, 8, and 10 h treatments. After 10 h treatment at 35°C, the mean mortalities for Sc, Hb, and Sf were 5.3%, 40.6%, and 70.3%, respectively. This is higher heat tolerance than the strains described by Morton and García-del-Pino (2009), which had at least 90% mortality after just 4 h of treatment at 35°C.

When treated at 40°C, the three species exhibited increasing mortalities (Sc<Hb<Sf) after both 1 and 2 h treatments, which is the same ranking of species' tolerance as with longer exposures to 35°C. This contradicts studies such as Susurluk and Ulu (2015) and Morton and García-del-Pino (2009), which suggested that Hb is more heat tolerant than Sc. These differences may be due to strain. Mortality of Sc was comparable to the mortality of the most tolerant isolates from the eastern USA, as Somasekhar et al. (2002) reported between 63 and 18% mortality for strains treated for 2 h at 40°C, and the same treatment caused 17.6% mortality in our study. Once again, the commercial strains we tested were more tolerant than those evaluated by Morton and García-del-Pino (2009), since they reported that only Hb retained minimal survival after 2 h exposure to 37°C, whereas in our tests all three species survived at least 1 h exposure at 40°C.

At 45°C, only Sc had minimal survival after 1 h treatment, and after all longer durations of treatment, mortality was 100% for all species. Most previous studies did not test EPNs at 45°C because complete mortality was reached at lower temperatures, although Ali et al. (2007) did record survival of Sc isolated from India as 55% after 6 h at 40°C and 50% after 6 h at 45°C. These differences could be due to strain.

The effects of product formulation on the survival of Sf exposed to high temperatures is limited. There was higher survival of Sf in powder formulation after 10 h at 35°C and higher survival of Sf in gel formulation after 1 h at 40°C, but for all other treatments there was no difference. These results suggest that growers do not need to consider product formulation when trying to choose the most effective EPN

product for environments with high temperatures. However, growers may have other reasons for choosing a particular EPN formulation, for instance, organic crop certification may exclude polyacrylamide gel products.

The effects of high temperatures observed in this study can help greenhouse growers to use EPNs more effectively. The commercial EPN products that we tested have heat tolerance that differs from published studies of wild isolated strains, so it is important to reference specialized reports. Although there is no consistent, significant impact of product formulation on EPN survival at high temperatures, EPN species has a significant effect. If greenhouse growers are applying Sf for pest control, they should consider switching to another species if soil temperatures surpass 35°C for at least 4 h, or reach 40°C for any duration. Survival of Sc will be unaffected by exposures of at least 10 h at 35°C, and survival will remain above 75% for up to 2 h at 40°C. EPN survival is minimal for all species at 45°C, and in growing environments with those conditions, alternative pest control will need to be considered.

Future research on commercial EPNs should test additional effects of product formulation, such as whether it impacts EPN survival during storage and shipment. It would also be helpful to further examine commercial EPN survival at high temperatures, with experiments conducted in actual greenhouses with even longer durations of exposure. This study provides a clear description of survival for exposures up to 10 h, but EPNs may be exposed to some high temperatures for days or weeks at a time.

REFERENCES

Abbott, W. S. 1925. A Method of Computing the Effectiveness of an Insecticide. J. Econ. Entomol. 3: 302–303.

Ali, S. S., R. Pervez, M. Abid Hussain, and R. Ahmad. **2007**. Effect of temperature on survival of Steinernema seemae, S. masoodi and S. carpocapsae (Rhabditida: Steinernematidae) and their subsequent infectivity to prepupa of Helicoverpa armigera (Hübner). Arch. Phytopathol. Plant Prot. 40: 183–187.

Cuce, E., D. Harjunowibowo, and P. M. Cuce. **2016**. Renewable and sustainable energy saving strategies for greenhouse systems: A comprehensive review. Renew. Sustain. Energy Rev. 64: 34–59.

Georgis, R., A. M. Koppenhöfer, L. A. Lacey, G. Bélair, L. W. Duncan, P. S. Grewal, M. Samish, L. Tan, P. Torr, and R. W. H. M. van Tol. **2006**. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control. 38: 103–123.

Grewal, P. S. 2000. Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida: Steinernematidae). Int. J. Parasitol. 30: 995–1000.

Jagdale, G. B., M. L. Casey, P. S. Grewal, and R. K. Lindquist. **2004**. Application rate and timing, potting medium, and host plant effects on the efficacy of Steinernema feltiae against the fungus gnat, Bradysia coprophila, in floriculture. 29: 296–305.

Kaspi, R., A. Ross, A. K. Hodson, G. N. Stevens, H. K. Kaya, and E. E. Lewis. **2010**. Foraging efficacy of the entomopathogenic nematode Steinernema riobrave in different soil types from California citrus groves. Appl. Soil Ecol. 45: 243–253.

Kaya, H. K., M. M. Aguillera, A. Alumai, H. Y. Choo, M. de la Torre, A. Fodor, S. Ganguly, S. Hazir, T. Lakatos, A. Pye, M. Wilson, S. Yamanaka, H. Yang, and R. U. Ehlers. **2006**. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol. Control. 38: 134–155.

Leite, L. G., D. I. Shapiro-Ilan, and S. Hazir. **2018**. Survival of Steinernema feltiae in different formulation substrates: Improved longevity in a mixture of gel and vermiculite. Biol. Control. 126: 192–197.

Morton, A., and F. García-del-Pino. **2009**. Ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean areas. J. Invertebr. Pathol. 102: 203–213.

Mukuka, J., O. Strauch, L. Waeyenberge, N. Viaene, M. Moens, and R. U. Ehlers. **2010**. Heat tolerance among different strains of the entomopathogenic nematode Heterorhabditis bacteriophora. BioControl. 55: 423–434.

Peters, A., R. Han, X. Yan, and L. G. Leite. **2016**. Production of entomopathogenic nematodes, pp. 157–170. *In* Microb. Control Insect Mite Pests from Theory to Pract. Elsevier Inc.

Shapiro-Ilan, D. I., S. Hazir, and L. Lete. **2015**. Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. J. Nematol. 47: 184–189.

Smits, P. H. 1996. Post-application persistence of entomopathogenic nematodes. Biocontrol Sci. Technol. 6: 379–387.

Somasekhar, N., P. S. Grewal, and M. G. Klein. **2002**. Genetic variability in stress tolerance and fitness among natural populations of Steinernema carpocapsae. Biol. Control. 310: 303–310.

Susurluk, I. A., and T. C. Ulu. **2015**. Virulence comparisons of high-temperature-adapted Heterorhabditis bacteriophora, Steinernema feltiae and S. carpocapsae. Helminthol. 52: 118–122.

CHAPTER 3

THE IMPACT OF HIGH TEMPERATURE EXPOSURE ON THE INFECTIVITY OF COMMERCIALLY AVAILABLE ENTOMOPATHOGENIC NEMATODES

Introduction

Entomopathogenic nematodes (EPNs) are used as biological control agents for soil-dwelling insect pests in agriculture and horticulture (Georgis et al. 2006). After entering an insect through natural openings, infective juveniles (IJs) release symbiotic bacteria that aid in killing and breaking down the host. (Lewis and Clarke 2012). The success of EPNs in controlling soil pests can be affected by numerous factors, including natural preferences for certain host species, the application rate of EPNs, and abiotic factors such as soil type, moisture, and high temperatures (Jagdale et al. 2004, Georgis et al. 2006, Kaspi et al. 2010, Shapiro-Ilan et al. 2015). Inefficient and expensive climate control within greenhouses makes high temperatures a significant concern during warmer growing seasons (Cuce et al. 2016). This series of studies sought to evaluate the impact of high temperature exposure and application rate on the infectivity of two commercially available EPN species, *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) (Sc) and *Steinernema feltiae* Filipjev (Sf), against the model host *Galleria mellonella* Linn (Lepidoptera: Pyralidae) and fungus gnats (*Bradysia impatiens* Johannsen; Diptera: Sciaridae).

Many previous studies have tried to elucidate the effects of temperature on EPN infectivity, each focusing on a subset of EPN species, strains, temperature ranges, durations of exposure, and hosts. Although a few studies have included commercially produced EPNs, most research has focused on wild isolates. Only a few studies have examined Sc and Sf together, and most of these have focused on model hosts instead of agricultural or horticultural target pests. Several studies have reported that the highest percent infection by both species occurs at 25°C (Grewal et al. 1994, Radová and Trnková 2010), but differences in high temperature tolerances can have a critical impact on EPN success in variable environments. These differences are summarized below.

Morton and García-del-Pino (2009) tested the infectivity of Mediterranean isolates, and observed Sc to be more heat tolerant than Sf. *G. mellonella* was infected by Sc at low levels at 32°C, while Sf could infect at only as high as 30°C. Grewal et al. (1994) studied infectivity of strains of Sc from the USA and Sf from Argentina and France. As in the previous study, the highest temperature for infection of *G. mellonella* by Sc was 32°C and the highest for Sf was 30°C.

Several studies described much higher heat tolerance for Sc than reported in the studies described above, which indicates that temperature tolerance can vary widely among strains of the same species. Testing one commercial strain of Sc and 14 strains isolated from the eastern USA, Somasekhar et al. (2002) treated IJs for 2 h at 40°C, and infection of *G. mellonella* was still between 42 and 79% after exposure to 1 IJ for 72 h. Ali et al. (2007) tested a strain of Sc isolated from India, treating at a range of high temperatures for 6 h. Surviving nematodes were applied to the prepupa of *Helicoverpa armigera*, and incubated for 3 days at 30°C. The highest infection of *H. armigera* by Sc was 90% at 25°C, then infection decreased to approx. 60% after treatment at 35°C, 50% after treatment at 40°C, and 40% after treatment at 45°C.

The success of pest control can also be affected by the application rate of EPNs, which is often chosen based on pest pressure and frequency of application. Varied doses of EPNs have been examined in several studies. For example, Addis et al. (2011) examined 4 Indonesian isolates of Sf, applying doses of 50 to 800 IJs to 40 *Tenebrio molitor* larvae in moist sand. The lethal concentration of IJs to kill 50% larvae ranged from 373 to 458 for the four strains. Jagdale et al. (2004) tested three application rates of commercial Sf against the fungus gnat, *Bradysia coprophila*, in pots of poinsettia, and the rates of 1.25, 2.5, and 5 $x10^5$ IJs/m^2 resulted in reduction of fungus populations by 9, 23, and 41% after 42 d, respectively. However, the reduction of fungus populations in pots of New Guinea impatiens were not significantly different after applications of 1.25 and 2.5 $x10^5$ IJs/m^2. These studies suggest that application rate can have an impact on EPN infectivity, but it is not always significant.

Our aim in this study was to examine the effect of high temperatures and duration of high temperature exposure on the infectivity of commercially available strains of Sc and Sf. Infectivity against both the model host *G. mellonella* and a common greenhouse pest, fungus gnats (*B. impatiens)*, was tested. The effect of three different application rates of Sc and Sf was also tested, by measuring differences in the emergence of adult fungus gnats. The results of this study will enable those making pest management decisions to have greater certainty when applying EPNs, especially as environmental conditions shift through the seasons.

Materials and Methods

Source of EPNs

Two commonly used species of EPNs, *Steinernema carpocapsae* (Sc) and *Steinernema feltiae* (Sf), were each provided by three biocontrol companies: BASF, E-nema, and Koppert Biological Systems, which were treated as three separate strains for each species. These companies use liquid culture for mass rearing of EPNs, and are the suppliers for most EPN distributors around the world. The companies vary in EPN product formulation; BASF and Koppert Biological Systems use gel-based preservation, while E-nema usually preserves EPNs in a powder-based formulation, which was included in this experiment. Products were sent with expedited shipping, and specialized packaging was used to preserve them at a cool temperature. Upon receipt of each shipment, products were placed in a refrigerator at 5°C for storage until use. The effect of high temperatures on EPN survival and infectivity was tested with the following three experiments.

Effect of Heat-treated EPNs on Galleria mellonella *Mortality*

Sublethal effects of high temperatures on EPNs were assessed by measuring infectivity, or percent host mortality, against the model host *G. mellonella*, with a five-day process. All three

commercial strains of Sc and Sf were tested. Products of Hb were not included in this experiment because of their prohibitively short storage period.

On the first day, EPNs were treated at high temperatures. A small sample of each EPN product was removed from the stored packages and allowed to warm up to room temperature. EPNs were then added to 25°C deionized water in 30 mL glass test tubes and diluted to approximately 2000 IJs/mL. Then, 0.25 mL of this solution was added to 0.75 mL water in glass vials (Kimble: 15x45 mm 1 Dram), either at 25°C for controls, or preheated to the treatment temperature of that setup day, resulting in 1 mL with approximately 500 IJIJs in each vial. These were kept in trays, and a separate tray was set up for each duration of exposure, each containing three vials for each combination of EPN species and strain.

The tray with control vials, which would receive 0 h treatment at high temperature, was placed directly into the 25°C incubator. The rest of the trays were placed in an incubator set to the single high temperature being tested that day, 30, 35, or 40°C, then transferred to the 25°C incubator after the allotted treatment duration. EPNs were treated for 8 h at 30°C and for 4 and 8 h at 35°C. Both species were treated for 1 h at 40°C, while only Sc received a 2 h treatment. These treatments were chosen based on EPN survival data (Fig. 2.2), so that infectivity could be checked for EPNs that had survived heat exposure. Temperature was monitored in the incubators using a digital data logger (Onset: HOBO UX100-003). Temperatures remained within 1°C of the target treatment temperature throughout each treatment period. Adding the 0.25 mL EPN solutions to preheated water in the vials helped to minimize the time it took to warm up to the target treatment temperature. Vials were provided an additional warm-up period in the treatment incubator (10, 15, and 20 min for 30, 35, and 40°C, respectively) in order to reach the target temperature before the timing for duration of exposure began. All trays remained in the 25°C incubator overnight, to allow EPNs time for recovery from heat shock, if needed.

On the second day, one sample (0.05 mL) was taken from each treatment vial, and EPN mortality was assessed. This data was compared to that of the EPN survival experiment in Chapter 2, to

ensure consistency of treatment effects between experiments. To take a sample, vials were lightly agitated until EPNs were evenly distributed by visual evaluation, then 0.05 mL was extracted with a pipette from the center of the water column. Drops were placed on a petri dish, examined under a dissecting microscope, and EPNs were counted as alive if they were actively moving, or if they moved after being gently rolled with a fine, flexible hair probe. To examine EPN mortality due to high temperature, the mortality in each sample was corrected based on that day's average control mortality for that EPN species and strain, according to Abbott's formula (Abbott 1925):

$$\text{Corrected proportion mortality} = \frac{\text{Mortality in sample} - \text{Average control mortality}}{1 - \text{Average control mortality}}$$

Two surviving EPNs were then transferred to each well of 24-well plates, which had already been filled with 1 g autoclaved fine sand (Quikrete: No. 1961) and 0.2 mL of deionized water. Surviving EPNs were arbitrarily selected and transferred with a fine hair probe under a dissecting microscope onto a water-soaked paper hole punch dot. The paper dot was then placed into a well, and the water film on the paper allowed for EPN dispersal into the well. This process ensured that EPNs were not injured during transfer.

Once all EPNs were transferred, one *G. mellonella* larva was placed in each well, lids were applied to the plates, and they were placed into the 25°C incubator. Each combination of EPN species, strain, temperature, and duration was tested against twelve larvae each test day. Twelve control wells were also set up on each test day for untreated EPNs of each species and strain against *G. mellonella*, as well as twelve control wells with *G. mellonella* but no EPNs, to assist in determining non-EPN larval deaths.

G. mellonella mortality was assessed after three days (± 30 min) of exposure to the EPNs. To evaluate mortality, motionless larvae were probed, and those that remained completely motionless were considered dead. There was minor *G. mellonella* mortality due to non-EPN causes, and those larvae were excluded from data analysis. This was determined by comparing to controls with no EPNs

and observing larval color change over several additional days, comparing to distinct color changes produced by EPN species. *G. mellonella* infected by Sc turn light pinkish-tan and those infected by Sf turn dark grayish-brown.

This experiment was repeated on three setup days per treatment temperature. The complete experiment was conducted a total of three times, with distinct production batches from the companies.

Effect of EPN Application Rate on Fungus Gnat Emergence

The effect of various application rates of EPNs on the emergence of adult fungus gnats (FG) was tested. This was partly in preparation for the experiment on the effect of heat-treated EPNs on the emergence of FG, to identify a single rate of untreated EPNs that would kill some, but not all, FG. FG larvae were obtained from a laboratory colony maintained with a procedure modified from Braun et al (2009).

Peat-based growing mix (Lambert: LM-3 All Purpose Mix) was moistened with tap water in a large tub, almost to the point of saturation. Clear plastic cups (Fabri-Kal: 12 oz. deli containers, product #PK12S-C) were filled with the mix to 4 cm depth and lightly patted down, then weighed to confirm they were in the same target mass range (120-135 g), ensuring the soil was moistened consistently. Three pieces of clear plastic straw were inserted vertically into the soil to later support a sticky card trap.

EPNs of one commercial strain of two different species, Sc and Sf, were diluted to 200, 1000, and 2000 IJs/mL, then 5mL of each mixture were pipetted evenly across the soil surface in each cup. This resulted in each cup receiving a total of 1000, 5000, or 10,000 IJs, from one of two species. Based on the surface area of soil in each cup, EPNs were applied at 12, 59, or 118 million IJs/100 m^2, which will be referred to as low, medium, and high application rates. Fifteen large, last-instar FG larvae were then transferred with a fine probe to the soil surface in each cup, and were observed to confirm they were still active after transfer. A polystyrene card coated with sticky adhesive on each side (Olson Products

Inc: 4 x 6 cm) was placed horizontally on top of the three straws to catch emerging FG, and the cups were closed with a mesh lid (TSK Supply: Fabric Waffle Lid).

Three replicate cups were set up for each combination of species and rate. Three controls with 15 FG larvae but no EPNs were also set up each day, to provide a standard to calculate proportion FG mortality due to EPN activity. Three cups were set up with no FG or EPNs, to ensure the soil was not unintentionally contaminated with FG, and no adults were ever found in these cups.

After addition of the FG larvae, cups were left for two weeks in a dark cabinet at approximately 25°C, to ensure that all surviving FG larvae had time to pupate and eclose. Adult FG that had been captured on each sticky card were counted. This process was replicated on three distinct setup days.

Effect of Heat-treated EPNs on Fungus Gnat Emergence

The effect of high temperature exposure on the infectivity of EPNs against FG larvae was assessed. Two commercial strains of both Sc and Sf were tested. FG were obtained from a laboratory colony, and maintained with a procedure modified from Braun et al (2009).

On the first day of the three-day process, cups were set up with moist growing mix and straw pieces using the same methods as in the application rate experiment, except the target mass range was 100 to 110 g per cup. Also, to minimize evaporation during treatment, the lids of cups were covered either with another cup, or a secured petri dish of the same diameter. Cups to be heat treated were placed in the appropriate incubator to preheat for 15 to 20 hours before EPNs were added. Cups that would have EPNs and 0 h heat treatment, and control cups with no EPNs were placed on a laboratory shelf at 24 to 27°C. Temperature was monitored in treatment incubators and the shelf using a digital monitor (HOBO UX100-003).

On the second day, all EPNs were diluted to 1000 IJs/mL in 80 mL glass beakers, using 25°C deionized water. Cups were removed from the incubator and 5 mL of the appropriate EPN solution were pipetted evenly across the soil surface, resulting in an application rate of approximately 59 million

IJs/100 m^2. Cups were immediately returned to the treatment incubator, at either 35 or 40°C for each setup day. At 35°C, Sc was treated for 8 and 12 h, while Sf was treated for 4 and 8 h. At 40°C, Sc was treated for 2, 4, and 6 h, while Sf was treated for 1 h. These temperatures and durations were chosen based on the results of previous experiments, to best clarify when heat could begin to affect EPN infectivity. Control cups with EPNs received 5mL of the appropriate solution, at the same time as the treatment cups, and were returned to the shelf. Controls cups with no FG or EPNs were also set up, to ensure the soil was not unintentionally contaminated with FG. No adults were ever found in these cups.

To allow cups to reheat to the target temperature, an additional warmup period was added to the treatments (15 and 20 min for 35 and 40°C, respectively). Each set of cups was removed from the incubator after the appropriate treatment duration, and placed on the same shelf as the control cups.

On the third day, large, last-instar FG larvae were selected from a lab-maintained colony and added to the treatment cups. After treatment cups cooled overnight, 15 larvae were added to the soil surface in each cup with a fine probe, except for the control cups with no FG or EPNs. Larvae were observed to confirm they were still active after transfer. A polystyrene card coated with sticky adhesive on each side (Olson Products Inc: 4 x 6 cm) was placed horizontally on top of the three straws to catch emerging FG, and the mesh lids were added again. All cups were then placed on the storage shelf.

After addition of the FG larvae, cups were left for two weeks, to ensure that all surviving fungus gnats had time to pupate and eclose. Adult fungus gnats that had been captured on each sticky card were counted. Average emergence in controls with no EPNs varied widely between setup days, and days with an average emergence of less than six FG were excluded from analyses, to ensure a more precise response variable.

This experiment was repeated on three to four setup days per treatment temperature. The complete experiment was conducted a total of three times, with distinct commercial production batches

Statistical Analysis

EPN mortality during the *G. mellonella* infectivity experiment was compared to EPN mortality from the survival experiment in Chapter 2, using a linear mixed effects model for each treatment temperature. The response variable was Abbott's corrected proportion of EPN mortality. The model for 30°C had fixed effects of species and experiment and their interaction. The models for 35°C and 40°C each had fixed effects of species, duration, and experiment, and their two and three-way interactions.

For the *G. mellonella* infectivity experiment, a generalized linear mixed-effect model of each treatment temperature, with a binomial distribution and a logit link, was fitted. The response variable was the count of alive versus dead *G. mellonella* after exposure to EPNs. Each model included fixed effects of EPN species and duration and their interaction.

For the application rate experiment, a linear mixed effects model was fitted, with the response variable of proportion emergence of FG compared to emergence in controls without EPNs. Each model included fixed effects of EPN dose, species, and their interaction, and the random effect of setup day.

For the experiment testing the effects of heat-treated EPNs on FG emergence, a linear mixed effects model for each combination of treatment temperature and EPN species was fitted. The response variable was the proportion emergence of FG, compared to emergence in controls without EPNs. Each model included the fixed effect of duration.

Each model also accounted for random effects of EPN strain, batch, and setup day. Posthoc tests were performed for each model, using Tukey's HSD to control for multiple comparisons. A residual analysis was performed for all linear mixed effect models, to check the model assumptions of normality and homogeneity. Analyses were performed with R studio (version 3.4.3).

Variability of data means is reported with the standard error.

Results

Effect of Heat-treated EPNs on Galleria mellonella *Mortality*

When comparing the proportion of EPN mortality during this experiment to that of the survival experiment in Chapter 2, there was no significant effect of experiment ($p>0.05$). Thus, the effects of high temperature treatment are comparable between the experiments.

The proportion of *G. mellonella* mortality varied with the treatments of applied EPNs (Fig. 3.1). Mortality caused by Sc was significantly higher than that of Sf at all durations for each temperature ($p<0.001$, for all comparisons). At 30°C, the mean mortality caused by Sc and Sf was 76.5 ± 2.4 and 45.9 ± 2.3%, respectively. This did not change significantly for Sc or Sf between 0 and 8 h exposure ($p=0.908$, $p=0.983$).

Mortality caused by Sc was unaffected by treatment at 35°C, even when comparing 0 h to 8 h exposure ($p=0.258$). Mortality caused by Sf at 35°C was significantly lower when treated at 4 h compared to 0h, falling to only 20.7 ± 3.4%, and even lower after 8 h compared with 4 h ($p<0.001$, $p<0.001$). *G. mellonella* mortality caused by Sc at 40°C was not significantly lower when treated at 1 h compared to 0 h, or 2 h compared to 1 h, but it was significantly lower after 2 h compared with 0 h, decreasing from 82.5 ± 2.3 to 69.9 ± 3.2% ($p=0.125$, $p=0.150$, $p=0.001$). Mortality caused by Sf at 40°C was significantly lower after 1 h treatment, compared to 0 h ($p<0.001$).

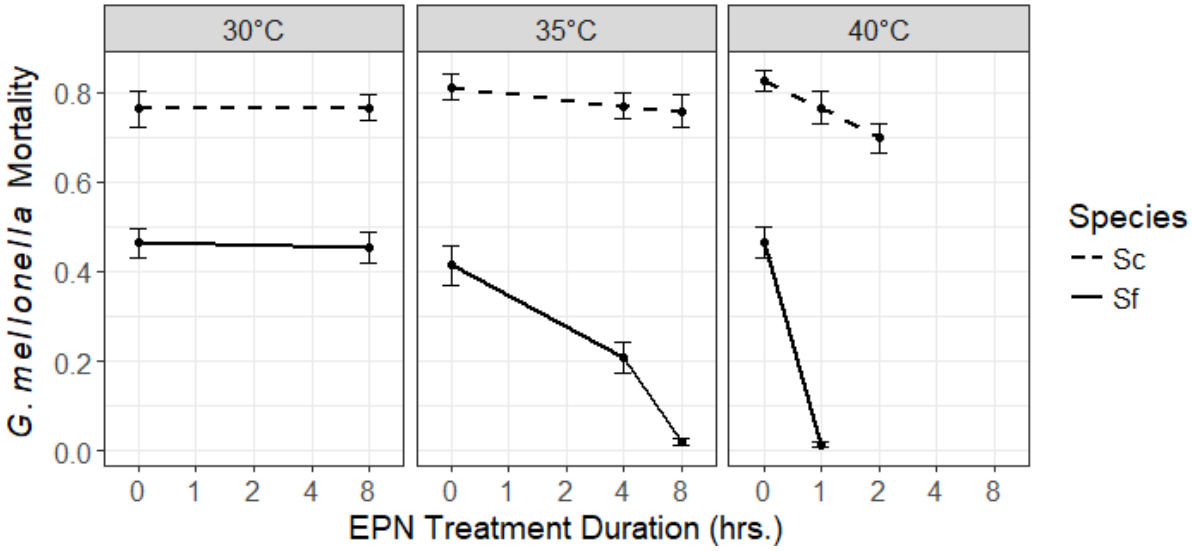

Figure 3.1. Mean proportion of *G. mellonella* mortality after three days exposure to one of two EPN species treated at various durations of three temperatures. Error bars represent +/- one standard error.

Effect of EPN Application Rate on Fungus Gnat Emergence

Proportion of FG emergence varied across treatments (Fig. 3.2). Both EPN species and application rate had a significant effect on FG emergence ($F_{1, 46}$=39.450, p<0.001; $F_{2, 46}$=25.352, p<0.001), but there was no significant interaction of these factors ($F_{2, 46}$=0.885, p=0.420).

There was significantly higher FG emergence after applications of Sc compared to Sf, with low, medium, and high application rates (p=0.002, p<0.001, p=0.006). Mean FG emergence after applications of Sc at low, medium, and high rates was 63 ± 4.9, 46 ± 6.2, and 24 ± 6.5%, respectively. There was no significant difference in FG emergence between low and medium rates of Sc, but there was a difference between medium and high rates (p=0.061, p=0.016). Mean FG emergence after applications of Sf at low, medium, and high rates and 39 ± 6.5, 11 ± 4.6, and 3 ± 2.0%, respectively. There was a significant difference in FG emergence between low and medium rates of Sf, but not between medium and high rates (p=0.002, p=0.531).

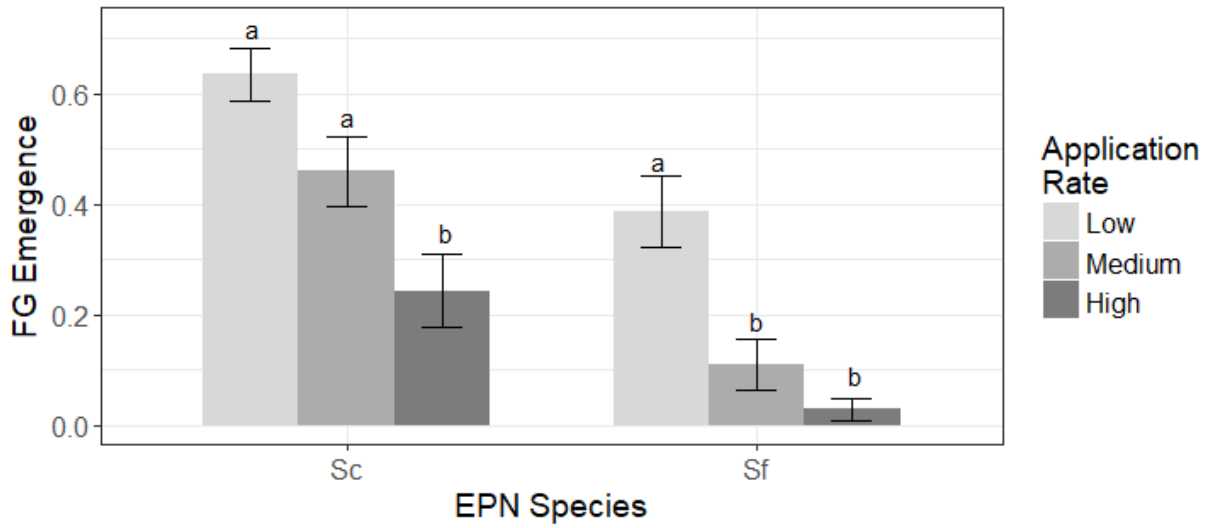

Figure 3.2. Mean proportion of FG emergence, compared to emergence in controls with no EPNs, after application of one of two EPN species at three different rates. Low, medium, and high rates were 12, 59, and 118 million IJs/100 m^2, respectively. Letters denote significant differences in emergence within treatments of the same EPN species. Error bars represent +/- one standard error.

Effect of Heat-treated EPNs on Fungus Gnat Emergence

The proportion of adult FG emergence varied with the treatments of applied EPNs (Fig. 3.3). Higher emergence indicates lower infection of FG larvae, whether caused by EPN mortality or reduced infectivity. There was no significant difference in FG emergence when Sc was treated at 35°C for 0, 8, or 12 h, with mean emergence across all durations of 42.5 ± 1.5% ($F_{2, 218}$=0.7165, p=0.490). Treatments of Sf at 35°C did affect FG emergence ($F_{2, 218}$=231.8, p<0.001), with a significant increase in emergence between 0 and 4 h treatments, and between 4 and 8 h treatments (p<0.001, p<0.001). The mean emergence was 7.0 ± 1.2, 36.6 ± 3.5, and 82.1 ± 3.1%, for the 0, 4, and 8 h treatments, respectively.

Treatments of both Sc and Sf at 40°C had a significant impact on FG emergence ($F_{3, 241}$=74.514, p<0.001; $F_{1, 109}$=876.43, p<0.001). There was no significant difference in emergence between treatments of Sc for 0 and 2 h, but there was a significant increase in emergence between treatments of Sc for 2 and 4 h, and between treatments of 4 and 6 h (p=0.523, p<0.001, p<0.001). The mean emergence was 41.5 ± 3.3, 73.3 ± 4.0, and 95.3 ± 3.0%, for the 2, 4, and 6 h treatments of Sc, respectively. There was a significant increase in FG emergence between treatments of Sf at 40°C for 0 and 1 h, as the mean emergence jumped from 6.8 ± 1.4 to 97.1 ± 2.9% (p<0.001).

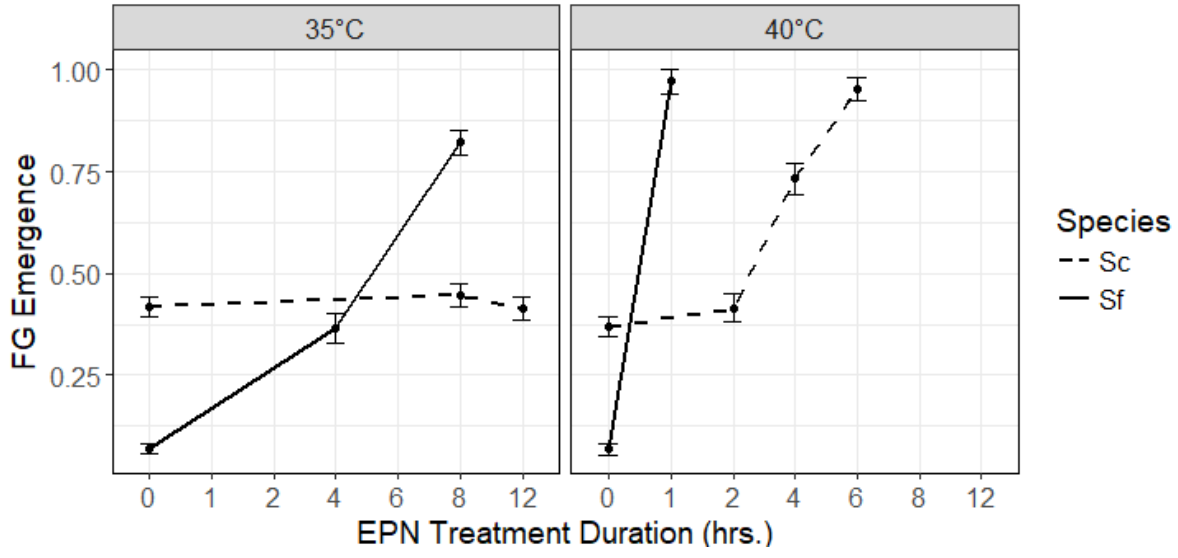

Figure 3.3. Mean proportion of FG emergence, two weeks after application of one of two EPN species treated at various durations of 35 or 40°C. Error bars represent +/- one standard error.

Discussion

EPN species, temperature, and duration of exposure can all have a significant impact on EPN infectivity against *G. mellonella* and FG. Treatment at 30°C had no impact on either Sc or Sf infectivity against *G. mellonella*, but exposure to higher temperatures did causes significant changes. Some of these effects were similar to that reported in previous studies, but there were many differences as well, likely due to varying high temperature tolerance among strains of each EPN species.

Exposure to 35°C did not affect the infectivity of Sc, but Sf infectivity was reduced against both *G. mellonella* and FG after 4 and 8 h treatments. After just 1 h exposure to 40°C, Sf did not cause any *G. mellonella* mortality or control FG emergence. Treatment of Sc for 2 h at 40°C slightly lowered the mortality of *G. mellonella*, and treatments of 4 and 6 h significantly reduced Sc control of FG.

These results are consistent with Morton and García-del-Pino (2009) and Grewal et al. (1994), which both report that Sc infectivity is less affected at high temperatures than Sf infectivity. However, our study suggests higher heat tolerance overall than strains of either species tested by Morton and García-del-Pino (2009) and Grewal et al. (1994), which reported that infection of *G. mellonella* did not occur after exposure to 35°C. Our results also contrast with the study by Ali et al. (2007), which reported that infectivity of Sc treated for 6 h at 35°C was reduced by approx. 30%, whereas treatments of 8 h at 35°C in our study did not significantly reduce Sc infectivity. The effects of treatment 40°C in our study, however, contrasted with Ali et al. (2007) in the opposite direction, as they reported that 6 h exposure only reduced Sc infectivity of *H. armigera* from 90 to 50%, while in our study the same treatment resulted in an increase of FG emergence from approx. 40 to 95%.

We did not evaluate infectivity of commercial strains at 45°C, but with such low EPN survival after just 1 h exposure as reported in Chapter 2, it is unlikely these strains would remain infective after any exposure.

Application rate of EPNs also had an effect on the adult emergence of FG, as a measure of larval mortality. Low, medium, and high rates, or 12, 59, and 118 million IJs/100 m^2, respectively, all resulted in reduced emergence compared to controls. There was significantly higher FG emergence with applications of Sc compared to Sf at all application rates, and FG emergence decreased as the rate increased for both species. For this reason, if FG are a target pest in a greenhouse, then it would be more effective to apply Sf than Sc, at least at low to moderate temperatures. Growers can choose from various application rates based on the pest pressure in their own production facility.

Populations of FG in our study seemed more reduced than by similar treatments in the study by Jagdale et al. (2004). Their application of commercial Sf at 50 million IJs/100 m^2 resulted in *Bradysia coprophila* populations at 60% compared to controls with no EPNs after 42 days, while in our study the application of Sf at 59 million IJs/100 m^2 resulted in *Bradysia impatiens* emergence at only 10% compared to controls. This could be due to a slightly higher application rate in our study, a difference in Sf preference for various FG species, or the effect of long vs. short-term reductions in FG populations.

Assays for infectivity against FG, both in the final experiment of this study and in future research, need to have low FG emergence in controls with untreated EPNs, but also have low enough EPN rates to be sensitive to changes in infectivity due to EPN heat treatment. Taking this into account, the medium rate of 59 million IJs/100 m^2, or 5000 IJs per cup, is ideal for FG assays with EPNs.

The effects of high temperatures on the infectivity of commercial EPNs reported above can help greenhouse growers ensure that their biocontrol agents will remain effective. If FG are a concern in a greenhouse and temperatures are well-regulated, growers may choose to initially apply Sf for biological control. However, the control of pests by Sf will decrease if soil temperatures surpass 35°C for at least 4 h or reach 40°C for any duration. If this situation is likely to occur, the grower should consider reapplying Sf when temperatures cool, or applying a different EPN species. Infectivity of Sc should remain high after at least 10 h exposure to 35°C, and up to 2 h at 40°C. While Sc is generally not as effective against FG

larvae as Sf at temperatures below 35°C, it will provide some control at temperatures that impair Sf Infectivity.

While the results of this study should be helpful to growers that deal with short-term spikes in greenhouse temperature, it would be helpful to have a description of the effect of high temperature on commercial EPN infectivity when exposed for days or weeks at a time. Future research should also include evaluations of commercial EPN infectivity against target pests such as western flower thrips pupae (*Frankliniella occidentalis* Pergande) and shore fly larvae (*Scatella spp.*).

REFERENCES

Abbott, W. S. 1925. A Method of Computing the Effectiveness of an Insecticide. J. Econ. Entomol. 3: 302–303.

Addis, T., M. Mulawarman, L. Waeyenberge, M. Moens, N. Viaene, and R. U. Ehlers. **2011**. Identification and intraspecific variability of Steinernema feltiae strains from Cemoro Lawang village in Eastern Java, Indonesia. Russ. J. Nematol. 19: 21–29.

Ali, S. S., R. Pervez, M. Abid Hussain, and R. Ahmad. **2007**. Effect of temperature on survival of Steinernema seemae, S. masoodi and S. carpocapsae (Rhabditida: Steinernematidae) and their subsequent infectivity to prepupa of Helicoverpa armigera (Hübner). Arch. Phytopathol. Plant Prot. 40: 183–187.

Braun, S. E., J. P. Sanderson, E. B. Nelson, M. L. Daughtrey, and S. P. Wraight. **2009**. Fungus gnat feeding and mechanical wounding inhibit Pythium aphanidermatum infection of geranium seedlings. Phytopathology. 99: 1421–1428.

Cuce, E., D. Harjunowibowo, and P. M. Cuce. **2016**. Renewable and sustainable energy saving strategies for greenhouse systems: A comprehensive review. Renew. Sustain. Energy Rev. 64: 34–59.

Georgis, R., A. M. Koppenhöfer, L. A. Lacey, G. Bélair, L. W. Duncan, P. S. Grewal, M. Samish, L. Tan, P. Torr, and R. W. H. M. van Tol. **2006**. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control. 38: 103–123.

Grewal, P. S., S. Selvan, and R. Gaugler. **1994**. Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. J. Therm. Biol. 19: 245–253.

Jagdale, G. B., M. L. Casey, P. S. Grewal, and R. K. Lindquist. **2004**. Application rate and timing, potting medium, and host plant effects on the efficacy of Steinernema feltiae against the fungus gnat, Bradysia coprophila, in floriculture. 29: 296–305.

Kaspi, R., A. Ross, A. K. Hodson, G. N. Stevens, H. K. Kaya, and E. E. Lewis. **2010**. Foraging efficacy of the entomopathogenic nematode Steinernema riobrave in different soil types from California citrus groves. Appl. Soil Ecol. 45: 243–253.

Lewis, E. E., and D. J. Clarke. **2012**. Nematode parasites and entomopathogens, Second Edi. ed, Insect Pathol. Elsevier.

Morton, A., and F. García-del-Pino. **2009**. Ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean areas. J. Invertebr. Pathol. 102: 203–213.

Radová, Š., and Z. Trnková. **2010**. Effect of soil temperature and moisture on the pathogenicity of two species of entomopathogenic nematodes (Rhabditida: Steinernematidae). J. Agrobiol. 27: 1–7.

Shapiro-Ilan, D. I., S. Hazir, and L. Lete. **2015**. Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. J. Nematol. 47: 184–189.

Somasekhar, N., P. S. Grewal, and M. G. Klein. **2002**. Genetic variability in stress tolerance and fitness among natural populations of Steinernema carpocapsae. Biol. Control. 310: 303–310.

CHAPTER 4

FINAL CONCLUSIONS

Species, temperature, and duration of exposure can all have a significant impact on EPN survival and infectivity, at various levels of these factors. Three commonly used commercially available EPN species, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) (Hb), *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) (Sc), and *Steinernema feltiae* Filipjev (Sf), were included in these studies. Treatments at 30°C had no impact on either the mortality of EPNs or their infectivity against *G. mellonella*. Some treatments at 35, 40, and 45°C, however, caused significant effects on EPN survival and infectivity, and described below.

Treatments at 35°C revealed a clear difference in heat tolerance of EPNs. There was higher mortality of Hb than of other species after 4 h treatment at 35°C, but then Sf mortality was highest after 8 and 10 h treatments. Mortality of Sc was lower than both Hb and Sf after 6, 8, and 10 h treatments. After 10 h treatment at 35°C, the mean mortalities for Sc, Hb, and Sf were 5.3%, 40.6%, and 70.3%, respectively. This is higher heat tolerance than reported for other strains examined in some separate studies. Infectivity of Sc was not reduced against *G. mellonella* after treatments up to 8 h at 35°C, or against FG after treatments up to 12 h, the longest treatments applied in these experiments. The infectivity of Sf against both *G. mellonella* and FG was reduced after both 4 and 8 h exposure to 35°C, even though 4 h treatment at 35°C had no impact on the survival of Sf. This was the only treatment in this study that had no effect on EPN survival, but did have sublethal effects resulting in lower infectivity.

When treated at 40°C, the three species exhibited increasing mortalities (Sc<Hb<Sf) after both 1 and 2 h treatments, which is the same ranking of species' tolerance as with longer exposures to 35°C. This contradicts several separate studies, which suggested that Hb is more heat tolerant than Sc. These differences may be due to strain. Survival of all three species at 40°C was observed to be higher than reported in previous studies. Treatments at 40°C also reduced the infectivity of Sc and Sf. After just 1 h

exposure to 40°C, Sf did not cause any *G. mellonella* mortality or control FG emergence. Treatment of Sc for 2 h at 40°C resulted in slightly lower mortality of *G. mellonella*, but there was no change in FG emergence. Treatments of 4 and 6 h at significantly reduced Sc control of FG.

At 45°C, only Sc had minimal survival after 1 h treatment, and after all longer durations of treatment, mortality was 100% for all species. Most previous studies did not test EPNs at 45°C because complete mortality was reached at lower temperatures. We did not evaluate infectivity of commercial strains at 45°C, but with such low EPN survival after just 1 h, it is unlikely these strains would remain infective after any exposure.

The results of this study can help greenhouse growers to make better pest management decisions as environmental conditions shift through the seasons. While product formulation did not have a consistent, significant effect on the survival of EPNs at high temperatures, EPN species and duration of exposure were significant factors. At the optimal temperature of 25°C there was higher *G. mellonella* mortality caused by Sc compared to Sf, but conversely, FG emergence was better controlled by Sf compared to Sc. Therefore, if FG are a concern in a greenhouse, growers may choose to initially apply Sf for biological control. However, the control of pests by Sf will decrease if temperatures spike and soil is at 35°C for at least 4 h or 40°C for at least 1 h. If this situation is likely to occur, the grower should consider reapplying Sf after temperatures cool, or applying a different EPN species. Infectivity of Sc should remain high after at least 10 h exposure to 35°C, and up to 2 h at 40°C. While Sc is generally not as effective against FG larvae as Sf at temperatures below 35°C, it will provide some control at temperatures that impair Sf infectivity. Infectivity of Hb was not specifically tested, but its high temperature tolerance was between that of Sc and Sf. EPN survival is minimal for all species at 45°C, so in growing environments with such extreme conditions, alternative pest control options will need to be considered.

These results should be made easily available to anyone who can use them. Ideally, researchers will have access through a scientific journal publication, and agricultural and horticultural professionals will have access through both an industry magazine publication and through a summary factsheet. For the latter two resources, it will be important to include a reminder of factors besides high temperature exposure that can affect the success of EPNs and other biological control agents.

The research on commercial EPNs in this study should be continued in multiple ways. Additional effects of product formulation, such as whether it impacts EPN survival during storage and shipment, should be tested. Similar experiments on EPN survival and infectivity at high temperatures should also be conducted in actual greenhouses, to ensure that our results are accurate outside of the laboratory. Longer durations of exposure should be tested in these greenhouse experiments, since EPNs may be exposed to some high temperatures for days or weeks at a time. Infectivity experiments should also be replicated with Hb, if tests can be conducted quickly enough to prevent degradation of the product during storage. Future research should also include evaluations of commercial EPN infectivity against target pests such as western flower thrips pupae (*Frankliniella occidentalis* Pergande) and shore fly larvae (*Scatella spp.*), both at optimal growing conditions and at high temperatures.