Jack Stevens

The Entomopathogenic Nematode, *Steinernema riobrave*, as a Biological Control for Mixed Billbug Infestations in Zoysiagrass

Introduction

Billbugs are a group of weevils in the genus Sphenophorus, which includes several species widely known for causing turfgrass disorders. In the Unites States, there are 64 species, with 11 recognized as pests. Four species — *Sphenophorus venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis* — are commonly found in Indiana turfgrass causing brown irregular patches in lawns, athletic fields, and golf courses (Duffy 304). Damage is caused either by adults as they feed and oviposit in stems, or by larvae which consume stems, crowns, stolons and roots (Doskocil 2045). Billbug damage is often misdiagnosed as drought, compaction, or disease (Duffy 304); however, their damage can be distinguished from other maladies by inspecting damage at the soil level. Here, grass is easily broken off due to feeding.

Management of billbugs is mostly carried out with chemical solutions, targeting the pest at different stages of infestation. A variety of insecticides including Pyrethroids, Organophosphates, Neonicotinoids, and Diamides may be used as preventative insecticides to kill overwintering adults prior to oviposition (Georgis 114). Early in an infestation, plant-systemic insecticides may be applied to target adults that are still active and early instar larvae that are feeding in the grass stems. Soil insecticides are used as a curative approach after damage is seen (Duffy 304). It is important to note that, while billbugs can be targeted later in an infestation, the best time to respond is before oviposition. Fewer insecticides are effective as systemic and soil insecticides compared to those that are used for preventative measures. (Richmond). Visual representations of the billbug life cycle and timing of applications would be helpful to communicate this information to growers — for whom it is most relevant — but they are presently lacking.

Because insecticide efficacy varies depending on the life stage(s) being targeted, understanding the life cycle of common billbug species is critical in their management. Generally, adults overwinter and emerge from thatch and surface litter in spring to mate and lay eggs in grass stems in late April and early May. Early instar larvae feed within the stems, moving to the crowns and roots in later instars. At this point, damage can be seen on the surface. By mid-summer, the larvae pupate, adults emerge in following weeks, and move to overwintering sites by late fall. *S. parvulus, S. minimus, and S. inaequalis* appear to be univoltine with overlapping adult activity (**Figure 1**), but the life cycle of *S. venatus* complicates the collective pattern when populations in an infestation are mixed (Duffy 307) (**Figure 2**). In contrast to the other three midwestern species, *S. venatus* overwinters in two cohorts, resulting in two rough peaks of adult activity (Doskocil 2049). One cohort — overwintering as larvae — emerges as adults in early summer, producing the first peak. Their offspring also overwinter as larvae (Duffy 309). The other cohort overwinters as adults and produces a generation that develops throughout summer. They emerge from August to October, creating the second peak, and overwinter as adults (Doskocil 2049). Because of this complicated cycle, determining the best insecticide for the correct life stage or stages becomes problematic in a mixed billbug infestation.

Alternatively, entomopathogenic nematodes (EPNs) have been used as a biological control in labs, fields, and greenhouses for a variety of insects including diaprepes root weevil, black pine weevil, fungus gnats, thrips, white grubs, billbugs, mole crickets, and sciarid flies.

Two families of EPNs — Steinernematidae and Heterorhabditidae — are available as biological controls to manage billbugs in turfgrass. *Steinernema carpocapsae* specifically has shown promise against billbug adults, suppressing about 78% of *S. parvulus* populations in a field study which was comparable to chemical standards (Georgis 114).

The basic life cycle of EPNs begins with infective juveniles (IJ) which seek-out and infect insect hosts (**Figure 3**). IJs in the families Steinernematidae and Heterorhabditidae carry symbiotic bacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively (Christen 889). Once the IJs locate a host, and enter via natural openings, they release their symbionts which overcome the host's immune response and kill it in 24-48 hours (Kaspi 243). The bacteria also defend the host from other invaders while providing the nematodes with the nutrients required for maturation and reproductive activity (Yu 91). 1-3 generations are supported within the host until nutrients are depleted or the space becomes overcrowded. At this, IJs are produced, and they exit the cadaver in search of a new host (Kaspi 243). Helpful graphic representations, again, are currently lacking in this area, though they would greatly benefit anyone learning about EPNs.

A relatively underused species of nematode, *Steinernema riobrave*, was first isolated from bollworm pupae in the Rio Grande Valley in the 1990s (Elawad 762) and has since shown promise as a virulent EPN for pest management. As it is important to pair species of nematode and pest, so it is also to use a species that is effective in the given environment. Thus, a major advantage of *S. riobrave* is its tolerance to heat and drought. Coming from the semi-arid tropics, this strain remains active in temperatures up to 35°C, with an optimal range of 25-30°C (Elawad 763). Given this, *S. riobrave* can be used in hotter, drier regions, or to supplement endemic nematodes in seasons of reduced activity (Duncan 184). These characteristics may also render *S. riobrave* more suitable for mid-summer curative billbug control where both the damage and high temperatures peak. In a study on *Diaprepes abbreviatus* in strawberry, the efficacy of *S. riobrave* was compared to that of imidacloprid. Renkema et al. found that 1-2 treatments of the EPN was as effective as the positive control, suppressing up to 90% of the pest populations (127). The authors of this study note that EPNs such as *S. riobrave* could be particularly useful for organic farming or as an easy-to-use biological control.

This capstone experience took place in two portions. First, a preliminary experiment was carried out with the objective to assess the potential effects of *S. riobrave* on mixed populations of billbugs in zoysiagrass as compared to *S. carpocapsae* and a chemical standard, Dylox 420 SL. Life cycle illustrations were also developed for bluegrass and hunting billbugs, and the entompathogenic nematodes that attack them. Their purpose was to highlight the stages of damage, and management tactics that could be used at different points during an infestation.

Materials and Methods

Rearing of S. carpocapse and S. riobrave

S. carpocapse and *S. riobrave* were purchased commercially and reared through one generation using *Galleria mellonella* larvae as the host. White's traps were used to collect infective juveniles for application in the field. IJs of each species were stored in a film of water and incubated in tissue culture flasks at 13°C until released into field plots.

To infect the *Galleria* larvae, filter paper was placed in petri dishes and wetted with either of the nematode solutions. Larvae were placed on the filter paper, divided evenly among the dishes. In the early stages of infection, after the larvae died and began to change color, they were transferred to White traps (White, 1927).

White's traps were made by inverting a small petri dish lid in the center of a larger petri dish to serve as a platform. Filter paper was placed on the lid, suspended above the bottom of the larger dish. When wetted with water, the edges sagged into the excess water at the bottom, while leaving the center elevated. *Galleria* larvae were placed on the elevated filter paper, covered, and incubated at room temperature until new IJs emerged. Emergence was monitored visually, and collection of the nematodes began when the larvae exuded a yellowish substance containing IJs. Once a day until IJs stopped emerging, the White traps were gently rinsed with water to drain the free nematodes into the solution at the bottom of the dish. This liquid was then transferred to labelled tissue culture flasks incubated at 13°C. Solution was stored in amounts of approximately 250mL which provided a shallow film of moisture in each flask for the nematodes to survive.

When estimating the density of nematodes in solution, the contents of all flasks were collected in a 1000mL beaker. After gently stirring to suspend the nematodes in the aggregate solution, 50mL was aliquoted for ease in the following step. Five 20uL samples were pipetted from the aliquot onto a microscope slide to be counted. This was repeated three times for each strain to yield the average density in their respective solutions.

Application on zoysiagrass

The trial was conducted in zoysiagrass located at Purdue University's Daniel H. Turfgrass Research and Diagnostic Center in mid-July. The stand of zoysiagrass was known to be infested with a mixture of at least 2 species of billbugs, S. parvulus and S. venatus. Sixteen plots, measuring 3x3ft, were arranged in a 4x4 square and separated by 1ft alleys. In each row, plots were randomly assigned one of 4 treatments: (1) control, (2) *Steinernema carpocapsae*, (3) *Steinernema riobrave*, and (4) Dylox 420 SL. 1 billion nematodes (n) per acre or 22,957n/ft^2 is the recommended rate of *Steinernema carpocapsae* (S.r.) but given a limited number of nematodes from the rearing process, a half rate of 11,478n/ft^2 or 103,300 nematodes per 3x3ft area was used here. *Steinernema riobrave* (S.r.) was applied at the same rate and Dylox was applied at the half rate .1mL/ft^2. The appropriate amounts of S.c., S.r., or Dylox were then aliquoted into 1.5 gallons of water and delivered evenly onto their respective plots using watering cans. Control plots were treated with 1.5 gallons of water via the same method.

Data Collection and Analysis

One week after the applications, plots were sampled using a 10.8 cm soil coring device to extract turf and soil cores to a depth of 7.62 cm. 3 samples — giving a total area of .3ft^2 — were collected from each plot, biased towards areas that appeared most damaged. The number of adults, pupae, and larvae were summed for each. The density of these stages and the density of total individuals were evaluated, using analysis of variance, and plotted in Excel using the data analysis ToolPak.

Life Cycle Illustrations

All illustrations were created in Adobe Illustrator and Photoshop. Detailed components such as beetles, larvae, leaves, and grass textures were first hand-drawn with pen and paper, photographed, and opened in Photoshop for initial editing. These components were then finalized as digital vectors in Illustrator, making them easy to use and alter in context. Background components and text were generated in Illustrator with the intent to be reusable but customizable for the specific life cycle.

Results

The results of the study provide general trends of treatment efficacy, though none were statistically significant given the scale of the experiment. The most common life stage found were larvae, totaling 33, followed by pupae with 16. Only 6 adult billbugs were recovered in the treated zoysiagrass plots: 5 in S.c., 1 in S.r., and none in control or Dylox (**Figure 4a**; $p = 0.270$). The number of pupae/.3ft^2 was highest in the control treatment, followed by S.c., S.r., and Dylox (**Figure 4b**; $p = 0.540$). The same trend was found for larvae/.3ft^2 (**Figure 4c**; $p = 0.340$), however, the trend is strongest when accounting for all life stages together (**Figure 4d**; $p = 0.191$).

Figures 1 and **2** were generated, illustrating the respective life cycles of bluegrass (*S. parvulus*) and hunting (*S. venatus*) billbugs. The figures also highlight potential management strategies based on the life stage present in an infestation. "Preventive" strategies refer to those used on adults prior to oviposition, "Early Curative" refers to systemic insecticides used on larvae feeding in the stems and crown, and "Late Curative" refers to soil insecticides once larvae have moved deeper to feed on roots. **Figure 3** shows the general life cycle of EPNs in the families Steinernematidae and Heterorhabditidae.

Discussion and Conclusion

Four species of billbug — *Sphenophorus venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis* — are common pests of turfgrass in Indiana, causing symptoms comparable to drought, compaction, and disease. Because the life stage(s) in an infestation occupy different regions of the plant and soil, it is critical to understand the billbug life cycle and where it is feeding when choosing the most appropriate insecticides. Furthermore, the life cycle of S. *venatus* is much different than that of the other three; thus, understanding which species is present can affect management decisions as well.

The illustrations developed should clarify some of these complexities as well as provide an easy supplemental reference when considering management options. In the current study, the billbug infestation being treated was mixed and the field experiment took place in mid-July. In the standard management of bluegrass billbugs (*S. parvulus*), this timing would call for a "Late Curative" approach since the late instar larvae present reside in the root zone. Both early and late instar larvae of hunting billbugs (*S. venatus*) are expected be present at this time, requiring "Preventive" and "Late Curative" insecticides. The infestation here, however, was treated with two species of EPNs (*Steinernema carpocapsae* and *S. riobrave*) and Dylox 420 SL, a chemical standard. Compared to the control, each treatment seemed to influence the total number of billbugs found one week after the application date: plots treated with S.c. contained the most billbugs, while those treated with Dylox had the fewest. Unfortunately, these results were not statistically significant, likely due to challenges with nematode rearing which resulted in modifications to the nematode application rate.

The ideal design of this experiment planned to use the full recommended rates of each EPN and insecticide in plots 5x5ft on both zoysiagrass and Kentucky bluegrass. During the rearing process, however, S.r. reproduced at much lower rates than expected and hardly yielded enough IJs for a single application at full-scale. In the time span allotted, the best option was to shrink the plot sizes and apply at a half rate on zoysiagrass only. The adjusted design was greatly limited in that half the number of replications, half the application rate, and a smaller application area were required. Further investigation should yield more significant trends when executed at full-scale.

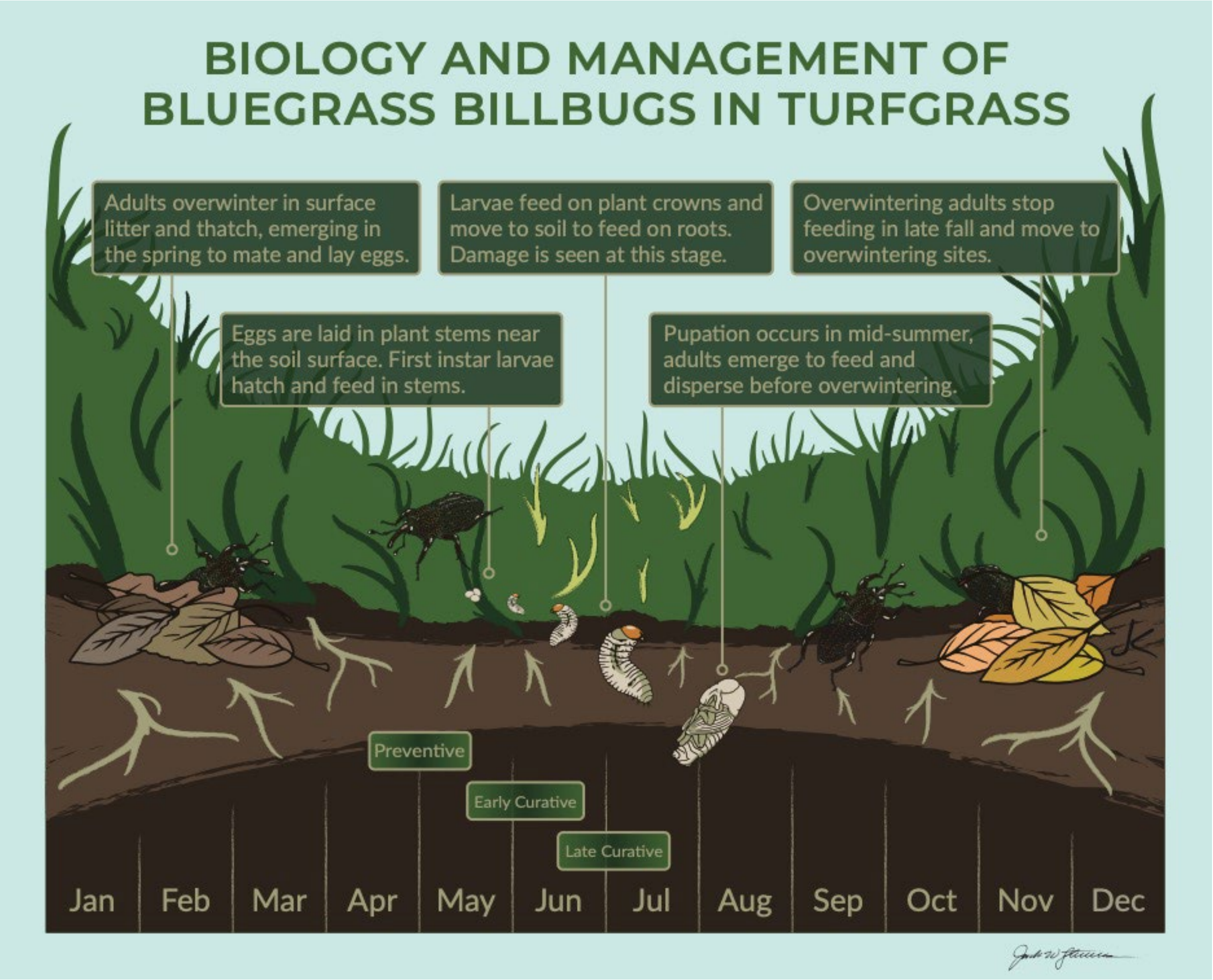


Figure 1 The life cycles of *Sphenophorus parvulus* (bluegrass billbug), *S. minimus, and S. inaequalis* are univoltine with overlapping activity. In spring, during the early stages of an infestation, "Preventive" insecticides can be applied to control adults before oviposition occurs. If eggs have already been laid, systemic insecticides — labelled "Early Curative" — should be used as early instar larvae will be feeding in the stems and crowns. After damage has been seen, larvae have moved into the root zone and soil insecticides must be applied as a "Late Curative" approach. Because there are fewer insecticides that are effective as systemic and soil insecticides, it is important to identify and treat an infestation early and with the proper chemical.

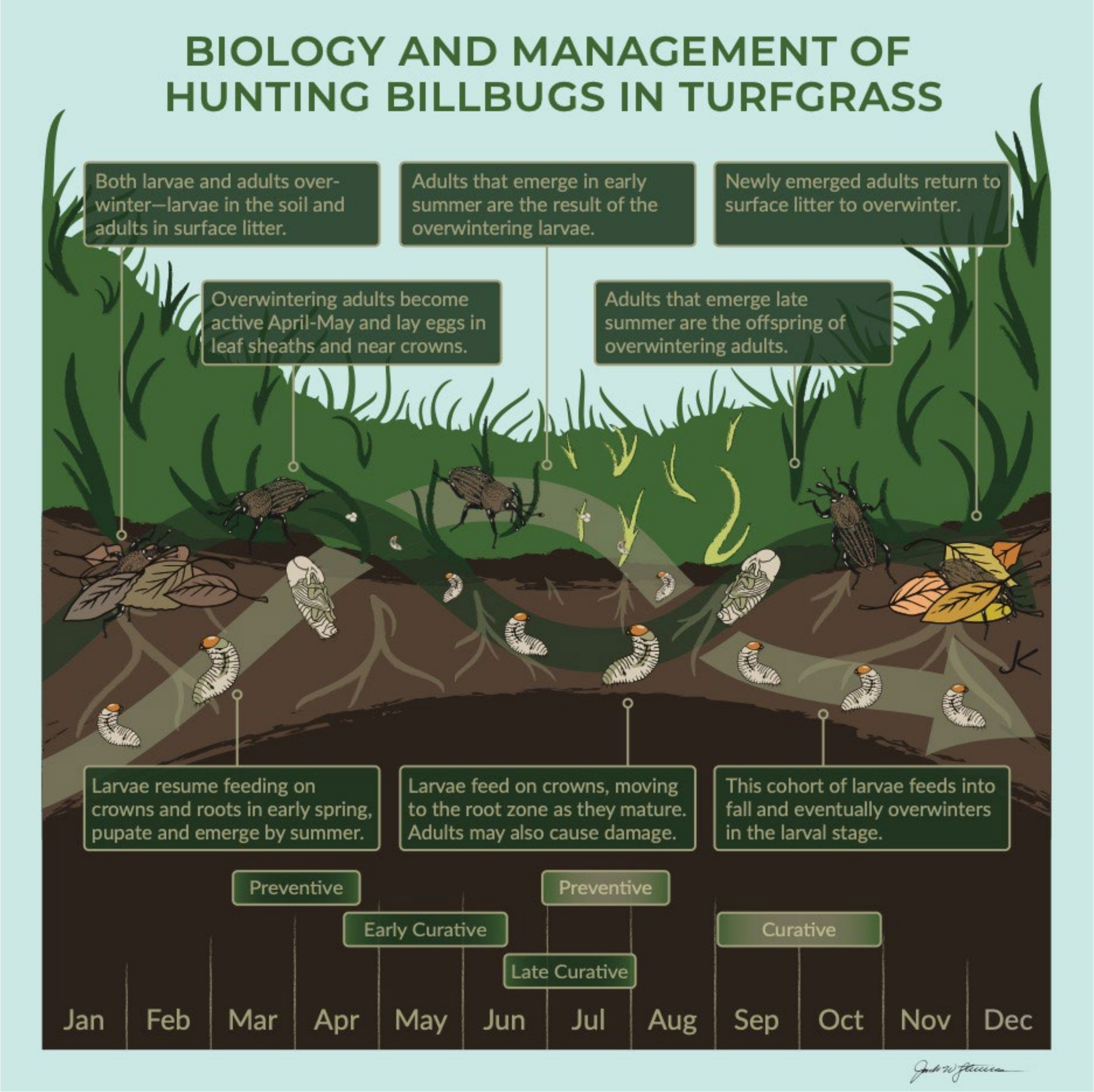
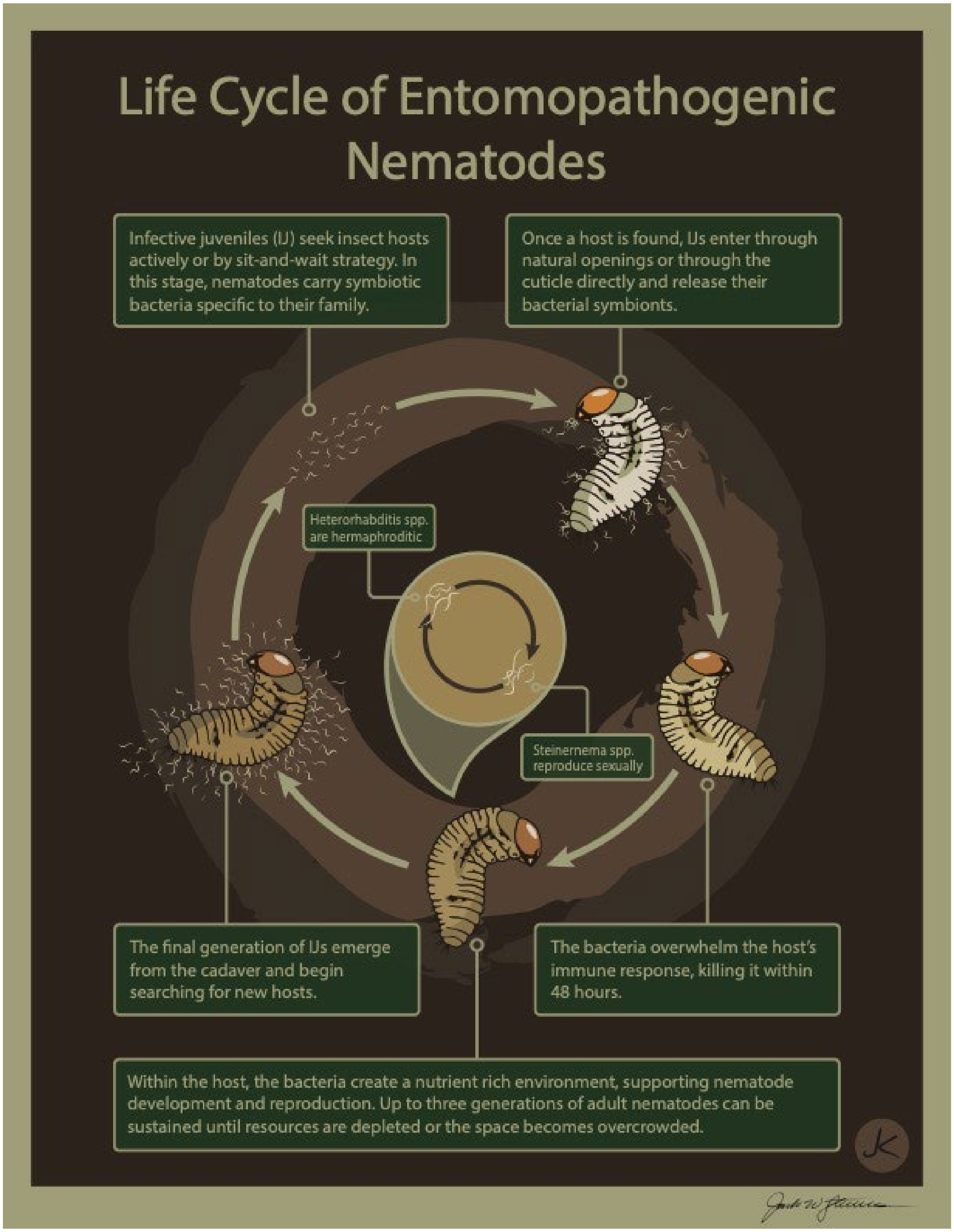


Figure 2 The life cycle of hunting billbugs, *Sphenophorus venatus*, can be confusing compared to those of *S. parvulus, S. minimus, and S. inaequalis*, but it is important to acknowledge when making management decisions. The green arrow, highlighting the cohort of billbugs which overwinter as adults, coincides with the dark green management strategies from March to July. Early in an infestation, "Preventive" insecticides are applied to control adults of this cohort before oviposition occurs, while systemic insecticides are used as an "Early Curative" option once their early instar larvae have begun feeding. Soil insecticides must be applied as a "Late Curative" approach if larvae have made it into the root zone. Similarly, the tan arrow highlights the cohort that overwinters as larvae. The control of this cohort corresponds with the management strategies in tan, offering Preventive and Curative options for the respective life stages.



Life Cycle of Entomopathogenic Nematodes

Infective juveniles (IJ) seek insect hosts actively or by sit-and-wait strategy. In this stage, nematodes carry symbiotic bacteria specific to their family.

Once a host is found, IJs enter through natural openings or through the cuticle directly and release their bacterial symbionts.

Heterorhabditis spp. are hermaphroditic

Steinernema spp. reproduce sexually

The final generation of IJs emerge from the cadaver and begin searching for new hosts.

The bacteria overwhelm the host's immune response, killing it within 48 hours.

Within the host, the bacteria create a nutrient rich environment, supporting nematode development and reproduction. Up to three generations of adult nematodes can be sustained until resources are depleted or the space becomes overcrowded.

Figure 3 The life cycles of two families of entomopathogenic nematodes, Steinernematidae and Heterorhabditidae, are described to provide users a better understanding of how they kill pests.

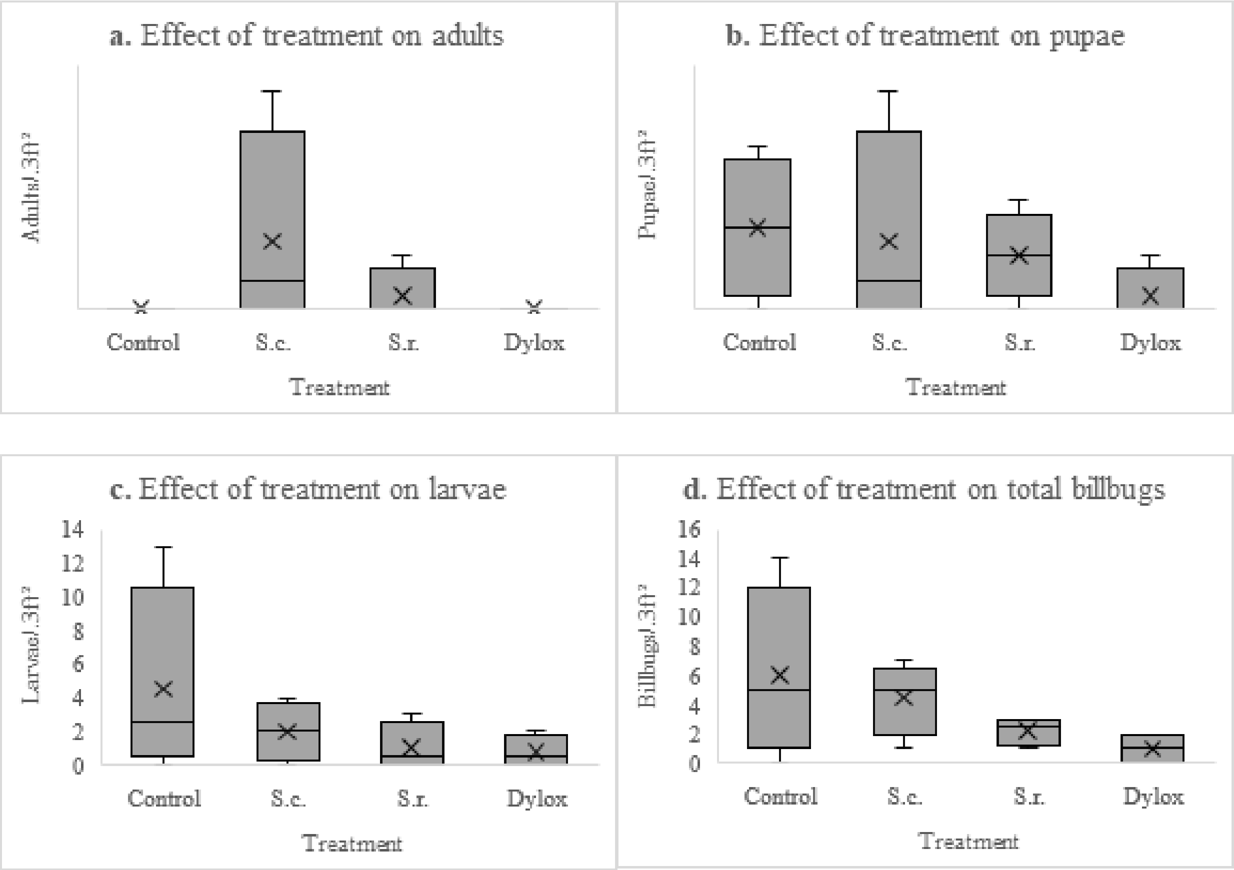


Figure 4 The number of billbugs sampled one week after insecticidal applications on zoysiagrass: control, S.c. (*Steinernema carpocapsae*), S.r. (*Steinernema riobrave*), and Dylox, a chemical standard. General trends of treatment efficacy are seen, with the number of pupae or larvae highest in the control, followed by S.c., S.r., and Dylox ($p = 0.540$, and $p = 0.340$ respectively), but no statistically significant relationships were produced. This trend is strongest when accounting for all life stages together ($p = 0.191$).

References

Christen, J. M., et al., "Responses of the entomopathogenic nematode, *Steinernema riobrave* to its insect hosts, *Galleria mellonella* and *Tenebrio molitor*." *Journal of Parasitology*, 134(6), 889–898. https://doi.org/10.1017/s0031182006002101.

Doskocil, J. P., & Brandenburg, R. L. (2012). "Hunting Billbug (coleoptera: Curculionidae) life cycle and damaging life stage in North Carolina, with notes on other billbug species abundance." *Journal of Economic Entomology, 105*(6), 2045–2051. https://doi.org/10.1603/ec12110

Duffy, A. G., Powell, G. S., Zaspel, J. M., Richmond, D. S. (2018). "Billbug (Coleoptera: Dryphthoridae: Sphenophorus spp.) Seasonal biology and DNA-Based life Stage Association in Indiana turfgrass." *Journal of Economic Entomology, 111*(1), 304–313. https://doi.org/10.1093/jee/tox340

Duncan, L. W., et al., "Incidence of Endemic Entomopathogenic Nematodes Following Application of *Steinernema riobrave* for Control of *Diaprepes abbreviates*." *Journal of Nematology*, 35(2), 178-186

Elawad, Sami A., et al. "The Life Cycle of *Steinernema abbasi* and *S. riobrave* in *Galleria mellonella*." *Journal of Nematology*, vol. 1, no. 7, 5 Oct. 1999, pp. 762–764., https://doi.org/10.1163/156854199508676.

Georgis, R., et al. "Successes and Failures in the Use of Parasitic Nematodes for Pest Control." *Biological Control*, vol. 38, no. 1, 2006, pp. 103–123., https://doi.org/10.1016/j.biocontrol.2005.11.005.

Kaspi, Roy, et al. "Foraging Efficacy of the Entomopathogenic Nematode *Steinernema riobrave* in Different Soil Types from California Citrus Groves." *Applied Soil Ecology*, vol. 45, no. 3, 28 Apr. 2010, pp. 243–253., https://doi.org/10.1016/j.apsoil.2010.04.012.

Renkema, Justin M., et al. "Control of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with *Steinernema riobrave* (Rhabditida: Steinernematidae) in Plasticulture Florida Strawberry." *Florida Entomologist*, vol. 104, no. 2, 2021, pp. 124–130., https://doi.org/10.1653/024.104.0208.

Richmond, Douglas. "Managing Billbugs in Turfgrass." *Extension Entomology*, Purdue University, https://extension.entm.purdue.edu/publications/E-266/E-266.html.

Yu, Hao, et al., "A Novel Strain of *Steinernema riobrave* (Rhabditida: Steinernemidae) Possesses Superior virulence to Subterranean Termites (Isoptera: Rhinotermitidae)." *Journal of Nematology*, 42(2):91-95, 2010

White GF. 1927. "A method for obtaining infective nematode larvae from cultures." Science 66: 302–303.