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Susceptibility of the Adult Japanese Beetle, *Popillia japonica* to Entomopathogenic Nematodes

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Abstract: To build upon prior research demonstrating the potential of entomopathogenic nematode dissemination by infected adult Japanese beetle, *Popillia japonica*, we evaluated susceptibility of the adult beetles to 20 strains of *Steinernema* and *Heterorhabditis* under laboratory conditions. The nematodes were applied at a rate of 10,000 infective juveniles per 10 adult beetles in 148 mL plastic cups containing autoclaved sand and sassafras leaves as a source of food for the beetles. All strains infected the beetles and caused 55% to 95% mortality. The most virulent strains that caused 50% beetle mortality in less than 5 days included a strain of *H. georgiana* (D61), three strains of *Steinernema* sp. (R54, R45, and FC48), and two strains of *S. carpocapsae* (All and D60). The ability of two strains of *Steinernema* sp. (R45 and R54) and two strains of *Heterorhabditis bacteriophora* (D98 and GPS11) to infect and reproduce in the beetle was further examined to assess the potential of infected beetles to disseminate nematodes upon their death. All four strains infected and killed the beetles, but only *Steinernema* strains reproduced in the cadavers. We conclude that both *Heterorhabditis* and *Steinernema* strains are able to cause mortality to adult Japanese beetle, but *Steinernema* strains may be effectively disseminated due to their reproduction in the beetle.

Key words: Heterorhabditis, *Steinernema*, biological control, Japanese beetle, *Popillia japonica*.

Since its discovery in the United States in 1916, the Japanese beetle (*Popillia japonica* Newman) has become a serious pest in urban landscapes. The beetles are gregarious and have generalist feeding habits, which result in a wide variety of plant species being defoliated. In early spring, over-wintering inactive grubs recover and feed on turfgrass roots until late spring. As adults, they emerge from the ground and seek out suitable food plants and begin to feed in late June. The adults that emerge earliest release a congregation scent which attracts other adults that emerge later. During the feeding period, mating begins quickly, and females intermittently leave plants after mating, burrow into the ground and lay eggs. About 95% of the eggs are generally laid by mid-August after which the eggs hatch, and the young grubs begin to feed on the root system. In late autumn, the grubs move deeper into the soil and remain inactive all winter (Potter and Held, 2002). Thus, in addition to above-ground damage, the grubs feed on grass roots, killing large patches of turf (Watschke et al., 1995; Potter, 1997). Altogether, the pest costs more than 450 million U.S. dollars in control annually in the United States (Potter and Held, 2002).

To manage the Japanese beetle, chemical insecticides such as carbaryl, chlorpyrifos and trichlorfon are used, although future use may be limited due to the continued implementation of the Food Quality Protection Act (FQPA), which places most of the carbamate and organophosphate insecticides currently in use to control Japanese beetle under scrutiny (Potter and Held, 2002). This, along with societal concern for public and environmental health, makes biological control an attractive alternative. In particular, entomopathogenic nematodes (EPNs) have received a lot of attention, especially for their use against soil-inhabiting pests (Grewal et al., 2005). Nematodes are attractive biological control agents because of their broad host range, ability to kill hosts quickly, and lack of any significant adverse effects on non-target organisms (Akhurst, 1990; Ehlers, 2005).

Entomopathogenic nematodes belonging to families Heterorhabditidae and Steinernematidae exist in soil in a type of resting form known as infective juveniles (IJs). The IJs carry symbiotic bacteria in their intestine: heterorhabditids are colonized by *Photorhabdus* and steinernematids by *Xenorhabdus* bacteria (Boemare and Akhurst, 2006). When the IJs invade a susceptible insect, they release the bacteria into the insect's hemolymph. The bacteria multiply in the hemolymph and produce a plethora of toxins, enzymes, antibiotics, and other biomolecules (An et al., 2009), killing the insect within 24-48 h and converting the cadaver into a food source suitable for nematode growth and reproduction. After 1-3 rounds of nematode reproduction, the bacteria recolonize the emerging IJs ensuring their transmission to a new insect host (Boemare and Akhurst, 2006).

The majority of research on EPNs and Japanese beetle has focused on beetle larvae (commonly referred to as grubs) as targets (Grewal et al., 2002; 2004; Grewal et al., 2005). Lacey et al. (1993) conducted a study on six strains of steinernematid and heterorhabditid nematodes against adult beetles, four of which caused significant mortality compared to the control. In a subsequent study Lacey et al. (1995) showed that beetles had the potential to disseminate EPNs both externally on their body and internally in the hemocoel. However, they concluded that additional research to identify more suitable nematodes is warranted. Therefore, the objectives of this study were to evaluate a broader range of nematode strains for their ability to infect, kill, and reproduce in the adult Japanese beetle.

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Materials and Methods

Stock cultures of nematodes: All nematode strains used in this study (Table 1) were reared in last instar *Galleria mellonella* (L.) larvae and harvested using White traps following the methods described by Kaya and Stock (1997). Eighteen of the twenty nematode strains used were collected from Ohio recently using the *G. mellonella* baiting technique (Bedding and Akhurst, 1975). *Heterorhabditis bacteriophora* GPS11 strain and *Steinernema carpocapsae* All strain were obtained from our laboratory stock cultures and reared as above.

Collection of the Japanese beetle: All Japanese beetles were collected from the campus of the Ohio Agricultural Research and Development Center (OARDC) in Wooster, Ohio, with Trecé Catch Can traps (Trece Inc., Adair, OK) using a feeding lure containing *PEG* (phenol ethyl propionate, eugenol, and geraniol). Beetles were stored for a maximum of 2 d in a cold room at 10°C prior to being used in the following experiments.

Pathogenicity of twenty nematode strains to adult Japanese beetle: Twenty nematode strains were divided randomly into two groups for ease of testing. The nematodes used and their test group designations are given in Table 1. Nematodes were applied to *P. japonica* adults at a rate of 1,000 infective juveniles (IJs) per beetle in 0.9 ml water in 148-mL plastic cups containing ten beetles and 9.1 g of play sand, which had been autoclaved at least two weeks prior to the experiment. This set up resulted in a total moisture content of approximately 10% in the cups. The cups were covered with lids to minimize moisture loss. Sassafras leaves were added daily as a food source for the beetles. Beetles challenged with only water served as controls. Each treatment including the control had four replicate cups. The cups were transferred to a growth chamber maintained at 22°C and no light. Beetle mortality was assessed every 24 hr for 7 d, and dead beetles were removed upon discovery. The procedure was repeated for a second group of ten nematode strains, and two trials were carried out for each test group. Frequency of dead individuals was analyzed using survival analysis in SAS (version 9.1), and differences between the control and the lowest performing strains were determined using the Log-rank statistic. LT50 (lethal time [d] to 50% mortality) and LT90 (lethal time [d] to 90% mortality) values were calculated using Probit analysis (Minitab version 15) and the differences among strains were based on the non-overlap of the 95% confidence intervals.

Infectivity of four nematode strains to adult Japanese beetle: To determine infectivity (i.e. rate of infection), adult Japanese beetles were kept in 148-mL plastic cups containing the nematodes at 22°C in dark conditions. Strains used were *H. bacteriophora* D98, *H. bacteriophora* GPS11, *Steinernema* sp. R54 and *Steinernema* sp. R45, which were randomly chosen from the twenty strains used in the previous experiment. Ten thousand IJs were applied in 0.9 mL water to cups containing 9.1 g of play sand, which had been autoclaved at least two weeks prior to the experiment. Ten beetles were then released in each cup. There were four treatments (strains) each with four replications (cups). Final moisture content of the sand was 10%, and the cups were covered with lids to minimize moisture loss. Containers were placed in a growth chamber at 22°C in darkness for 7 d. Sassafras leaves were provided daily as a food source. Beetle mortality was assessed every 24 hr for 7 d, and cadavers were removed and stored at 5°C for approximately one

Table 1. Pathogenicity of entomopathogenic nematode *Heterorhabditis* and *Steinernema* species and strains against adult Japanese beetle as measured by % mortality 7d after exposure, LT50 and LT90 (lethal time [d] to 50% and 90% mortality, respectively) values. SE = standard error of the mean; CI = 95% confidence interval.

Species	Strain	Test Group	% Mortality	LT50(d)	SE	CI	LT90(d)	SE	CI
H. bacteriophora	GPS11	1	78.8	5.4	0.2	5.0-5.8	9.0	0.5	8.3-10.1
H. bacteriophora	D2	2	90.0	6.3	0.4	5.6-7.3	13.0	1.4	10.9-16.8
H. bacteriophora	D54	2	88.8	6.3	0.4	5.7-7.2	12.0	1.1	10.4-14.8
H. bacteriophora	D99	1	63.8	6.5	0.2	6.1-7.0	9.9	0.5	9.0-11.2
H. bacteriophora	D17	1	53.8	7.6	0.5	6.8-8.9	13.0	1.2	11.1-16.3
H. bacteriophora	D58	2	85.0	6.5	0.4	5.8-7.6	13.1	1.4	11.0-16.9
H. bacteriophora	D29	2	73.8	8.6	0.8	7.3-11.0	15.9	2.1	12.8-22.1
H. bacteriophora	R96	2	90.0	5.9	0.4	5.3-6.9	12.7	1.3	10.7-16.3
H. bacteriophora	D98	1	65.0	6.5	0.2	6.1-7.0	10.0	0.6	9.1-11.4
H. georgiana	D61	2	97.5	3.8	0.3	3.2-4.4	11.1	1.1	9.3-14.3
H. georgiana	D64	2	82.5	6.8	0.4	6.2-7.7	11.8	0.9	10.3-14.2
Heterorhabditis sp.	F30	2	85.0	5.6	0.5	4.8-6.9	14.6	2.1	11.7-21.3
S. carpocapsae	All	1	77.5	4.3	0.2	3.9-4.7	8.7	0.5	7.9-10.0
S. carpocapsae	D60	2	93.8	4.6	0.3	3.9-5.3	12.4	1.4	10.2-16.5
Steinernema sp.	D86	1	72.5	5.0	0.2	4.5-5.4	9.7	0.6	8.7-11.2
Steinernema sp.	R95	1	60.0	7.4	0.4	6.7-8.5	13.8	1.2	11.9-17.0
Steinernema sp.	FC48	1	78.8	4.4	0.2	4.0-4.8	8.8	0.5	8.0-10.0
Steinernema sp.	R54	1	90.0	4.1	0.2	3.8-4.4	7.5	0.3	6.9-8.3
Steinernema sp.	R45	1	82.5	4.3	0.2	3.9-4.7	8.4	0.5	7.7-9.5
Steinernema sp.	D59	2	81.3	6.1	0.8	5.0-8.5	18.1	3.6	13.3-32.4

month, until they could be dissected. Three dead beetles were selected randomly from each replicate and dissected to confirm the presence of nematodes and to count the number of nematodes per cadaver and to record the life stage (juvenile or adult) of each nematode to determine the extent of development. The experiment was repeated. The data were analyzed with a randomized complete ANOVA (SAS version 9.1) by trial and pooled. The total number of beetles killed varied between 60 and 76 (out of 80 tested) among treatments. The treatment factor was the nematode strain, and data were analyzed for normality and homogeneity of variance. Data were transformed using ln(x+1), and mean separations were done using Tukey's test at $P \leq 0.05$.

Reproduction of four nematode strains in adult Japanese beetle: To determine reproductive ability of EPNs, the beetles were infected using the infectivity procedure described above. Briefly, 10,000 IJs were applied in 0.9 ml water to 148-mL plastic cups containing ten beetles and 9.1 g of autoclaved play sand. There were four nematode treatments (*H. bacteriophora* D98, *H. bacteriophora* GPS11, *Steinernema* sp. R54, and *Steinernema* sp. R45) each with three replications. The cups were covered with lids to minimize moisture loss. Containers were placed at 25°C in darkness for 5 d to allow for infection to occur. After 5 d, dead beetles from each replicate were removed and placed on White traps at 25°C for 17 d in order to collect emerging IJs using methods described by Kaya and Stock (1997). The average total number of IJs emerged per beetle was determined by counting. The experiment was repeated. The total number of infected beetles that were placed on the white traps from the two trials ranged between 50 and 60 (out of a total of 60 treated) for different strains. Data were analyzed with a randomized complete ANOVA (SAS version 9.1), by trial and pooled. Data were checked for normality and homogeneity of variance and transformed using ln(x+1), and mean separations were done using Tukey's test at $P \leq 0.05$.

Results

Pathogenicity of twenty nematode strains to adult Japanese beetle: All nematode strains tested caused mortality of the adult *P. japonica* (Table 1). Even the lowest performing nematode strains (*H. bacteriophora* D17 and *H. bacteriophora* D29) from the first and second groups of strains, respectively, caused significantly higher mortality than the control (χ^2 = 27.0154, df = 1, p = 0.0001 for group one; χ^2 = 18.5486, df = 1, p = 0.0001 for group two). From this, it can be inferred that all nematode strains tested caused significantly higher mortality than the control. In the first group of strains, six strains caused more than 70% mortality, 7 d after treatment, compared to the control mortality of 15% ± 0.05%. In this group, the nematode strains that caused the highest mortality were *Steinernema* sp. R54 (90%), *Steinernema* sp. R45 (83%), and *Steinernema* sp. FC48 (79%) and *H. bacteriophora* GPS11 (79%) (Table 1). In the second group, all ten strains caused more than 70% mortality, 7 d after treatment, compared to the control mortality of 41% ± 0.07%. The nematode strains that caused the highest mortality were *H. georgiana* D61 (98%), *S. carpocapsae* D60 (94%), *H. bacteriophora* D2 (90%), and *H. bacteriophora* R96 (90%). Although control mortality was high at 7 d after treatment, the results were similar even at 2 and 3 d after treatment when the control mortality was only 3% ± 0.01% and 10% ± 0.01%, respectively, for the first group of strains and 5% ± 0% and 16% ± 0.02%, respectively, for the second group of strains. The Probit analysis performed on the corrected mortality data revealed that *H. georgiana* D61, *Steinernema* sp. R54, *Steinernema* sp. R45, *S. carpocapsae* All, *Steinernema* sp. FC48, and *S. carpocapsae* (D61) had LT50s values of less than 5 days (Table 1). The amount of food eaten by beetles was not measured, although both the treated and control beetles appeared to eat until death.

Infectivity of four nematode strains to adult Japanese beetle: Of the two heterorhabditids, *H. bacteriophora* GPS11 had higher infectivity as determined by the number of nematodes penetrated per beetle, with an average of 38 nematodes per beetle, compared to *H. bacteriophora* D98, which had 5 nematodes per beetle (Fig. 1). No adult heterorhabditids were found in beetle cadavers in either trial, although the cadavers showed typical signs of infection by *Photorhabdus* bacteria. Of the two steinernematids, *Steinernema* sp. R45 had an average of 146 nematodes per beetle and *Steinernema* sp. R54 had 179 nematodes per beetle. The majority of the steinernematids found inside the beetle cadavers were adults. There was no significant difference between the two steinernematid strains for the numbers of adults found in the beetles ($P \leq 0.05$).

Reproduction of four nematode strains in adult Japanese beetle: Among the four strains tested, the two *Steinernema*

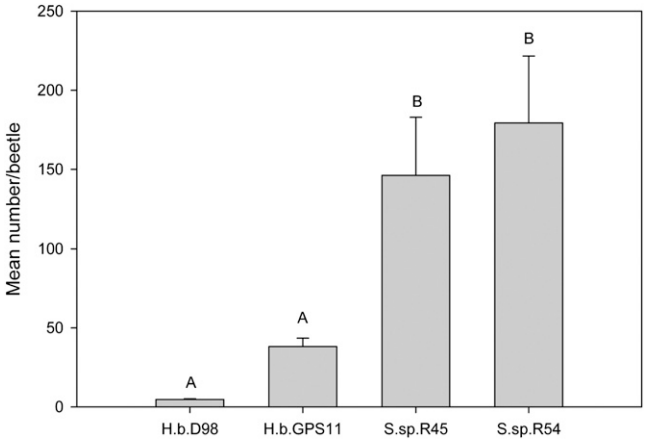

Fig. 1. Average number of entomopathogenic nematodes *Heterorhabditis bacteriophora* strains D98 and GPS11, and unidentified *Steinernema* sp. strains R45 and R54 found in dissected adult *Popillia japonica* cadavers in an assessment of infectivity of nematode strains.

strains produced significantly more offspring in the infected beetle (n = 30) than the two *Heterorhabditis* strains ($P \leq 0.05$). *Steinernema* sp. R54 produced an average of 2,423 IJs per beetle, while *Steinernema* sp. R45 produced an average of 1,958 IJs per beetle. One of the three replicates of *H. bacteriophora* D98 produced 127 IJs per beetle, while the other two replicates produced none. *H. bacteriophora* GPS11 did not reproduce in any of the replicates (Fig. 2).

Discussion

Our findings demonstrate that, in addition to the larval (grub) stage, adult Japanese beetles are significantly affected by all strains of EPNs tested. In a study conducted by Trdan et al. (2008), *S. feltiae*, *S. carpocapsae*, *H. bacteriophora*, and *H. megidis*, were applied to adult flea beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae) at a range of rates and temperatures. It was found that *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora* would be suitable for controlling adult flea beetles during warm summer months. *S. feltiae* was the most effective treatment, when 483 to 1467 IJs/adult beetle were used. Within the family Scarabaeidae, a study showed that adult Taro beetles (*Papuana uninodis*) were susceptible to *S. glaseri* in laboratory when nematodes were applied at a rate of 10^6 IJs/m^2, although a field test with adult beetles did not result in damage reduction (Theunis, 1998). Lacey et al. (1993) investigated the use of EPNs against adult Japanese beetles, which showed that *S. carpocapsae*, a *Heterorhabditis* sp., *S. feltiae*, and *S. glaseri* caused significant mortality in adult beetles compared to the control. Additionally, Lacey et al. (1995) found that *S. glaseri* penetrated and killed about 45% of the *P. japonica* adults within 4 days, following a treatment with 10,000 nematodes per 20 beetles, and that beetles infected with *S. glaseri* produced an average of 238 ± 50.1 IJs/adult. Our findings together with previous research suggest that there is potential for the control of the adult Japanese beetle using EPNs.

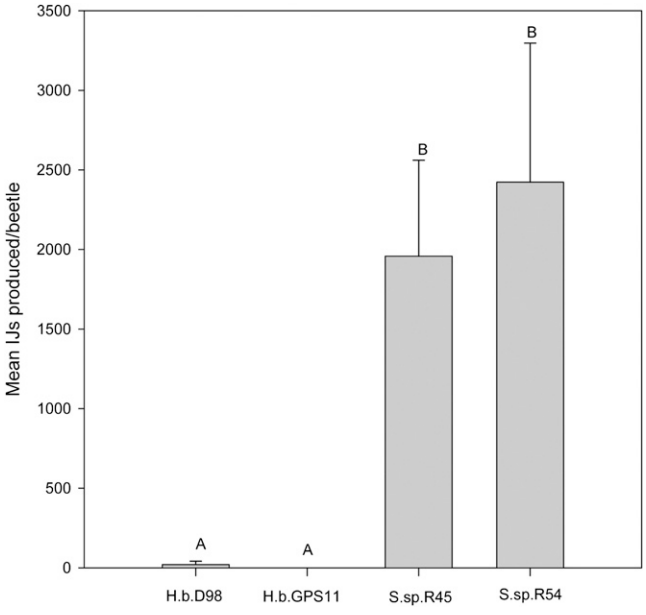

Fig. 2. Reproduction of entomopathogenic nematodes *Heterorhabditis bacteriophora* strains D98 and GPS11, and unidentified *Steinernema* sp. strains R45 and R54 in adult *Popillia japonica* cadavers after being held for 17 d at 25°C.

H. georgiana D61, *Steinernema* sp. R54, *Steinernema* sp. R45, *S. carpocapsae* All, *Steinernema* sp. FC48, and *S. carpocapsae* D61 had LT50 values of less than 5 days. These results show that EPN species such as *S. carpocapsae* that are relatively ineffective for controlling white grubs (Cui et al., 1993), can be highly virulent against the adult beetles indicating significant stage specific differences in insect susceptibility to nematodes. To be suitable for autodissemination via infected adult beetle, however, a given nematode species must provide excellent control of the grubs. Among the strains included in this study, the susceptibility of *P. japonica* grubs to only *H. bacteriophora* GPS11 and *S. carpocapsae* All strain has been evaluated (Grewal et al., 2002). Therefore, the susceptibility of the grubs to all other nematode strains tested in this study should be evaluated. Nevertheless, the high susceptibility of adult Japanese beetle to *S. carpocapsae* is interesting and may be related to the nematodes' ambush forging behavior and general adaption to highly mobile and surface dwelling insects (Campbell and Gaugler, 1993; Grewal et al., 1994). As *S. carpocapsae* is more desiccation tolerant and has better storage stability than heterorhabditid nematodes (Grewal and Peters, 2005) it may be more suitable for placement in the outdoor pheromone traps and be disseminated by the beetles for inoculative release into difficult to reach areas against a wide variety of pest insects.

Another important finding of this study was that, unlike steinernematid nematodes, which penetrated beetles, developed into adults, and reproduced, heterorhabditid nematodes were not able to fully complete their life cycle following penetration. It is possible that the adult *Heterorhabditis* nematodes were not found in dissected beetles in Experiment II due to slower development, as the infected cadavers were stored at 5°C as soon as they were discovered. However, cadavers in the reproduction study (Experiment III) were constantly kept at 25°C, but similar inhibition of the *Heterorhabditis* life cycle was observed. This may suggest that the Japanese beetle adults are not suitable for *H. bacteriophora* reproduction. Wang et al. (1995) found that despite a similar ability of *S. glaseri* and *H. bacteriophora* to cause mortality to *P. japonica* grubs, IJs of *S. glaseri* were able to tolerate the gut fluid and avoid host's immune response, while IJs of *H. bacteriophora* exhibited poor tolerance to gut fluid and induced a strong host immune response. A similar phenomenon may occur in adult beetles against *H. bacteriophora* IJs, although

bacteria may have been released prior to the death of the nematodes, which this study did not address. Another possibility is that the adult Japanese beetle cadavers are nutritionally unsuitable for the development and reproduction of heterorhabditids. In a study of lipid- and protein-based supplements in *Tenebrio molitor* diets, Shapiro-Ilan et al. (2008) found that nutritional content of an insect host diet can affect host susceptibility to EPNs and nematode fitness. Further work should be done to elucidate the reason(s) behind the poor reproduction of heterorhabditids in adult Japanese beetles.

Based on these results, EPNs show promise for the control of adult *P. japonica*. Future work should include development of methods of bringing Japanese beetles into contact with nematodes under field conditions. One idea being explored is the modification of existing pheromone traps as "infestation stations" in which adult beetles could get infected with EPNs. Such a trap could provide a microenvironment that could protect EPNs from the harsh environmental conditions that currently restrain EPNs from being used as foliar sprays. Additional research should also be done on the ability of infected beetles to pass nematodes on to other beetles while mating or to grubs in the soil. In this "autodissemination approach," not only would infected beetles eventually die, but they would introduce EPNs into the environment as means of augmentative biological control. Prior work by Klein and Lacey (1999) included an inoculation chamber containing the entomopathogenic fungus *Metarhizium anisopliae* added to a modified Japanese beetle pheromone trap. Beetles were able to pick up the fungus, which caused significant beetle mortality. Such a trap might be modified for use with EPNs. Based on our findings we conclude that, in an approach to simply infect adult Japanese beetles, either *Heterorhabditis* spp. or *Steinernema* spp. may be effective, although if autodissemination is the goal, steinernematids seems to be the better candidates for further investigation.

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