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Association of *Ovavesicula popilliae* (Microsporida: Ovavesiculidae) With Winter Mortality of Larvae and Reduced Fecundity of Female Japanese Beetles (Coleoptera: Scarabaeidae)

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ABSTRACT Populations of Japanese beetle at sites in Michigan where *Ovavesicula popilliae* (Andreadis) was introduced in 1999 and 2000 were compared with nearby control sites from fall of 2005 through spring of 2008. Percent infection by *O. popilliae* and winter mortality of Japanese beetle were determined by sampling larvae in October and April from 12 golf holes on six courses in southeast Michigan and eight holes on four courses in southwest Michigan. Adult Japanese beetles were also collected from these golf courses in July and August of 2007 to determine the impact of *O. popilliae*-infection on egg development in females. In southeast Michigan, *O. popilliae* appeared to spread rapidly from the 100 m^2 plots where it was previously introduced to surrounding golf course holes between 2000 and 2006. However, data from southwest Michigan suggests that *O. popilliae* had already been introduced into the area. Regression analysis of data from all 20 golf course holes gives a significant relationship between percent infection of larvae with *O. popilliae* and winter mortality of Japanese beetle. Mean winter mortality of larvae around golf course holes where <10% were infected with *O. popilliae* was 24.7% compared with 41.7% mortality where 10–30% were infected, and 72.0% mortality where >30% were infected. Females infected with *O. popilliae* contained 50% fewer mature eggs than uninfected females. In addition, females from golf courses where all of the fairways and roughs were treated annually with imidacloprid contained 48% fewer mature eggs than females from golf courses where insecticides were only used on the fairways or not at all.

KEY WORDS *Popillia japonica*, *Ovavesicula popilliae*, introduction, turfgrass, golf courses

The Japanese beetle, *Popillia japonica* Newman, is one of the most destructive pests in eastern North America (Fleming 1972). More than $450 million is spent each year for frequent insecticide applications to blueberries, grapes, golf course fairways, nurseries, homelawns, gardens, and for renovating or replacing damaged turf and ornamental plants (Potter and Held 2002). Japanese beetle is the most destructive pest of golf course turfgrass in the eastern United States. Insecticide use has become routine to prevent direct injury to turfgrass from larval consumption of roots, or secondary injury caused by skunks, raccoons, or birds foraging for Japanese beetle larvae (Vittum et al. 1999). In the urban landscape, the Japanese beetle is a serious flower, fruit, and shade tree pest. It frequently defoliates roses, lindens, sycamores, Japanese maple, birch, chestnut, sassafras, hibiscus, crabapple, ornamental cherries, mountain ash, grapes, raspberries, and many other plants (Fleming 1972). Homeowners and gardeners are often forced to choose between abandoning susceptible plants or making frequent insecticide applications between June and September.

Discovered in southern New Jersey in 1916, the Japanese beetle slowly spread across most of the Eastern and Midwestern United States over the next 80 yr, becoming established as far west as Missouri, Kansas, and Minnesota by 2000 (Hadley and Hawley 1934, Fleming 1968, USDA 2000). In the first 20 yr after the Japanese beetle was discovered 38 species of parasitoids and predators were collected from Japan or South Korea and introduced into the United States, but by 1968 only *Tiphia vernalis* Rohwer, *Tiphia popilliavora* Rohwer, and *Istocheta aldrichi* (Mesnil) could be regularly found (Fleming 1968). Although instances of a high rate of parasitism have been reported for each of these parasitoids, their distribution is localized, and they do not appear to be providing adequate control of Japanese beetle (Vittum et al. 1999, Potter and Held 2002).

Entomopathogenic microbes were not a part of the foreign exploration for natural enemies of Japanese beetle and none were intentionally introduced. However, some pathogens may have arrived with the original infestation of Japanese beetle, or with live Japanese beetle larvae that were brought to the United

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States in the 1920s to introduce parasitoids (Fleming 1968). Several pathogens of Japanese beetle were discovered in New Jersey between 1929 and 1935. Two of these were believed to be important enough to initiate a program of mass production and introduction into other states: *Steinernema glaseri* (Steiner), an entomogenous nematode; and *Paenibacillus popilliae* (Dutky), the milky disease bacterium. Initially both of these pathogens were believed to provide excellent long-term control of Japanese beetle within 5 yr of introduction. However, by 1968, *Steinernema glaseri* was described as being an important pathogen only under suitable environmental conditions, and success of the *P. popilliae* program was being questioned because outbreaks of Japanese beetle were observed in many locations where *P. popilliae* had been previously established (Fleming 1968, Dunbar and Beard 1975, Potter and Held 2002). More recent surveys of pathogens of Japanese beetle indicate that the average rate of infection of Japanese beetle larvae by *Paenibacillus* spp. at any one point in time is 3.7% in Connecticut and 1.0% in Michigan (Hanula and Andreadis 1988, Cappaert and Smitley 2002, Dingman 2008). Recent taxonomic work by Dingman (2008) on *Paenibacillus* pathogens of white grubs suggests that it is more appropriate to refer to them as *Paenibacillus* spp. unless a molecular analysis is used for species identification. Although an average of 3.7% infection and occasional epizootics contribute to natural control, some researchers believe that *P. popilliae* has lost virulence over the last 30 yr (Hutton and Burbutis 1974, Dunbar and Beard 1975, Klein and Jackson 1992, Klein 1995, Redmond and Potter 1995).

After 1950, no new parasitoids or pathogens of Japanese beetle were introduced into the United States, and very little research was published on the regulation of populations by natural enemies. In 1986, a survey of pathogens of Japanese beetle in Connecticut revealed that 25% of all larvae were naturally infected by *Ovavesicula popilliae* Andreadis, a previously unknown pathogen, found in the malpighian tubules of Japanese beetle (Hanula and Andreadis 1988). After 3-yr of an in-depth field study, Hanula (1990) concluded that *O. popilliae* may be important in regulating populations of Japanese beetle, because at two of four study sites the density of Japanese beetle larvae declined rapidly as the incidence of *O. popilliae*-infected larvae increased from 40 to 80%. *Paenibacillus* spp., was also active at the same sites, making it difficult to interpret the relative importance of each pathogen or their interaction.

O. popilliae spores are produced in sporophorous vesicles found in the malpighian tubules of infected larvae and adults (Hanula 1990). Infections, which are initiated when Japanese beetle larvae ingest spores in the soil, are almost entirely restricted to the malpighian tubules, although in rare cases sporophorous vesicles may be found in fat bodies. The infection of adults appears to be transstadial: *O. popilliae* infections of larvae continue through pupation into the adult stage, where sporophorous vesicles can also be found in the malpighian tubules (Vossbrink and Andreadis 2007).

Based on the promising work of Hanula (1990) and Hanula and Adreadis (1990), distribution of *O. popilliae* and *Paenibacillus* spp. in Michigan was investigated in 1999 and 2000 by examining Japanese beetle larvae from 35 locations in 15 counties of southern Michigan (Cappaert and Smitley 2002). *O. popilliae*, reported to be widespread and epizootic in Connecticut, was absent at 33 of 35 locations in Michigan, and *Paenibacillus* spp. or nematodes were found in <1% of the larvae examined (Cappaert and Smitley 2002). In addition, *Tiphia vernalis* Rohwer and *Istocheta aldrichi* (Mesnil) were not found at any of the 35 locations sampled (Cappaert and Smitley 2002).

In this paper we report the introduction of *O. popilliae* to sites in Michigan in 1999 and 2000, and the incidence of *O. popilliae*-infection of Japanese beetle from 2005 through 2007 at sites where it was introduced and at nearby control sites. The potential impact of *O. popilliae* on populations of Japanese beetle was investigated by determining the survival rate of Japanese beetle larvae from October to April at sites with varying levels of *O. popilliae* activity, and by examining the relationship of *O. popilliae* infection of females to their fecundity.

Materials and Methods

Introduction of *O. popilliae* in 1999 and 2000. A total of 1,850 live Japanese beetle larvae were collected from sites in Massachusetts where *O. popilliae* was active in September, 1999. Live larvae were held in individual paper cups with 20 cm^3 of sterilized moist soil for transport to Michigan in coolers. A subsample of 50 larvae was taken and the larvae dissected to provide an estimate of percent infection by *O. popilliae.* Within 4 d of being collected in Massachusetts, 450 live larvae were introduced into a 100 m^2 research plot in the irrigated rough at each of two golf course sites in southeast Michigan and southwest Michigan (a total of four introduction sites and 1,800 larvae).

Larval Sampling. From fall of 2005 to spring of 2008 we returned to the same golf courses and to six additional control golf courses where *O. popilliae* was not previously introduced. At each golf course Japanese beetle larvae were sampled in early to mid October and again in mid April to determine population density, infection levels, and to estimate winter mortality rates. Each Japanese beetle population that was sampled (replicate) consisted of the larvae in the irrigated Kentucky bluegrass (*Poa pratensis L.*) rough on both sides (within 5.0 m) of a single golf course fairway. A total of 20 golf course holes were sampled: four holes on two golf courses in southwest Michigan located within 500 m of where *O. popilliae* was introduced in 1999 and 2000; four holes on two control golf courses in southwest Michigan that are >5 km away but <15 km away from an introduction site; four holes on two golf courses in southeast Michigan located within 500 m of where *O. popilliae* was introduced in 1999 and 2000; and eight holes on four control golf courses in

Table 1. List of golf course holes (n = 20 sites) where Japanese beetle larvae and adults were collected to determine infection by *O. popilliae*

Site no.	Region of Michigan	*O. Popilliae* introduction	Golf course	Insecticide use	Hole	*O. popilliae* percent infection (n)			
						Oct. 05/ Apr. 06 larvae	Oct. 06/ Apr. 07 larvae	July/Aug. 07 adults	Oct. 07/ Apr. 08 larvae
1	SE[a]	Yes	Willow Metropark	Some fairways	9	7.9 (79)	21.3 (92)	4.5 (164)	14.5 (62)
2	SE	Yes	Willow Metropark	Some fairways	10	6.3 (40)	5.1 (63)	—	—
3	SE	Yes	Orchard Lake CC	All fairways and roughs	3	1.3 (94)	0 (56)	7.2 (180)	—
4	SE	Yes	Orchard Lake CC	All fairways and roughs	4	18.1 (42)	20.5 (90)	—	27.8 (41)
5	SE	No	Pine Valley GC	Some fairways	4	2.1 (124)	8.1 (124)	3.5 (120)	—
6	SE	No	Pine Valley GC	Some fairways	Sod field	3.2 (74)	1.9 (108)	—	—
7	SE	No	Pine Lake CC	All fairways and roughs	13	− (3)	0 (23)	0 (120)	—
8	SE	No	Pine Lake CC	All fairways and roughs	16	− (7)	0 (57)	—	—
9	SE	No	Bloomfield Hills CC	All fairways and roughs	13	0 (28)	− (0)	0 (60)	—
10	SE	No	Bloomfield Hills CC	All fairways and roughs	16	5.6 (36)	− (5)	—	—
11	SE	No	Cracklewood GG	Some fairways	4	2.1 (45)	0 (37)	0.7 (150)	—
12	SE	No	Cracklewood GG	Some fairways	6	4.1 (72)	0 (48)	—	—
13	SW	Yes	Medalist GC	Tees and greens only	4	11.1 (17)	13.6 (32)	6.6 (180)	7.7 (13)
14	SW	Yes	Medalist GC	Tees and greens only	5	7.1 (28)	6.1 (33)	—	—
15	SW	Yes	Eastern Hills GC	All fairways, no roughs	5	16.0 (25)	20.7 (40)	13.9 (180)	—
16	SW	Yes	Eastern Hills GC	All fairways, no roughs	7	24.1 (40)	6.3 (51)	—	16.7 (12)
17	SW	No	Binder Park GC	All fairways, no roughs	18	38.5 (70)	63.7 (25)	12.8 (156)	—
18	SW	No	Binder Park GC	All fairways, no roughs	6	33.9 (46)	51.9 (21)	—	13.6 (22)
19	SW	No	Kalamazoo CC	All fairways and roughs	15	14.3 (21)	46.2 (52)	10.0 (180)	—
20	SW	No	Kalamazoo CC	All fairways and roughs	1	10.9 (55)	20.0 (65)	—	—

Site locations are mapped in Fig. 1.
[a] SW = southwest, SE = southeast.

southeast Michigan that are >5 km away but <15 km away from an introduction site (Table 1; Fig. 1). Insecticide application was a common practice used for maintaining turfgrass on all golf courses, but no insecticide was applied to our sampling sites.

Larvae were sampled from around each hole by cutting turf squares (324 cm^2 and 6 cm deep) located along a line running parallel with the edge of the fairway, but 2.3 to 4.6 m into the irrigated rough. A turf square was dug every 6 m along this line, until eight turf squares were dug on both sides of a fairway, for a total of 16 turf squares per hole. Each turf square was removed to examine the roots and soil for the presence of Japanese beetle larvae. Larvae were placed individually into paper cups containing 50 cm^3 of moist soil. All of the cups with larvae from one turf square were then placed into a sealable plastic bag and the bag labeled for plot identification. Plastic bags containing cups with larvae were then placed into coolers for transport to the laboratory where they were immediately frozen in water, one larva per glass vial. Larvae were collected in early October of 2005, 2006, and 2007, and in mid April of 2006, 2007, and 2008. When turf squares were dug in April, they were also located 2.3 to 4.6 m into the irrigated rough on the same holes that were sampled in October in previous year, but the location of the turf squares was off-set by 1–2 m to avoid digging in the same square where larvae had been removed in October.

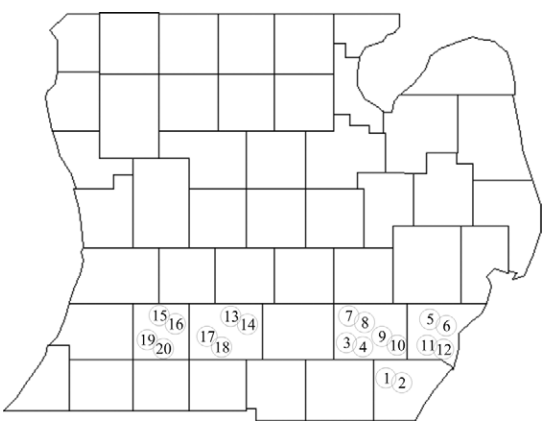

Fig. 1. Location of research sites listed in Table 1. Two consecutive numbers located close together (1 and 2, 3 and 4, etc.) indicate two different holes at the same golf course.

Diagnosis of Infection by *Paenibacillus sp.* and *O. popilliae*. Within 6 mo of collecting and freezing larvae they were individually thawed in the laboratory. Within 30 min of thawing, the integument of each larva was punctured on the prothorax just behind the head and a drop of hemolymph extracted and placed on a clean glass microscope slide. The hemolymph sample from each larva was examined for the presence of *Paenibacillus* spp. sporangia. Bacterial rods that were ≈5 μm-long, swollen at one end, and contained a spore or a spore plus a parasporal body, were considered to be *Paenibacillus* spp. (Cantwell 1974; Tanada and Kaya 1993; Dingman 2008, 2009). In fall of 2005, the first year of this study, the entire 10 μl hemolymph sample under a 2.25 cm^2 cover slip was searched for the presence of *Paenibacillus* spp. sporangia, sometimes requiring 20 min per slide. Based on the number of *Paenibacillus* spp. sporangia found, larvae were classified as being not infected (<10 sporangia found), having a trace of infection (10–100 sporangia found), or as infected (>100 sporangia

Table 2. Incidence of *O. popilliae* infection of Japanese beetle larvae at golf courses in Michigan in Oct. 2005, 2006, and 2007 at sites where it was introduced in 1999 and 2000, compared with infection rates at nearby control golf courses[a]

Treatment	n^b	Sites in Table 1 and Fig. 1	*O. popilliae* infection level (%)				
			1999[c]	2000	2005	2006	2007
O. popilliae introduction sites in southeast Michigan	4	1–4	0	0	3.5 ± 4.1 A[d]	20.3 ± 19.8 B	20.8 ± 19.6 A
Control sites in southeast Michigan	8	5–12	0	0	0 ± 0 B	1.2 ± 1.9 C	1.4 ± 3.9 B
O. popilliae introduction sites in southwest Michigan	4	13–16	0	6.5	16.8 ± 18.8 A	16.0 ± 20.2 B	10.8 ± 9.9 A
Control sites in southwest Michigan	4	17–20	0	10.0	29.8 ± 15.4 A	52.8 ± 14.9 A	22.3 ± 20.9 A

[a] Data are means ± SD.
[b] Replicates are a golf course hole, with two holes being sampled from each golf course.
[c] Only two sites of each treatment sampled in 1999 and 2000.
[d] Introduction site means are not significantly different from control means when both are followed by the same letter (Tukey's HSD or Tukey-Kramer, $P = 0.05$).

found). In subsequent years, each hemolymph sample was examined for 5 min and larvae were classified as not infected (<100 sporangia found) or infected (>100 sporangia found).

After a hemolymph sample was removed and examined, each larva was then dissected under a stereoscope and 3–4 malpighian tubules were excised and mounted on a glass microscope slide in a drop of normal saline for examination under a phase-contrast compound microscope. Larvae were considered to be infected with *O. popilliae* if the malpighian tubules contained spheroid to ovoid-shaped raspberry-like sporophorous vesicles, each containing 32 spores (Andreadis and Hanula 1987).

Adult Sampling. The population of adults near the two holes where we sampled larvae on each golf course were sampled with standard Japanese beetle traps baited with floral lures (phenylethyl propionate, geraniol, and eugenol, 3:7:3) and pheromone (Trécé, Salinas, CA). Two traps were placed 100 m apart, between the two holes. Because adults may fly >100 m to traps, beetle catches from the two traps were combined as a sample of the adults on both holes. Although no insecticide was applied to the turf in our research plots at any site, insecticide was sometimes used on the same fairway and rough just outside of our plots. Because of concern that insecticide use could affect the catch of adults in our traps or the fecundity of females, we asked the superintendents at each of the 10 golf courses to provide us with insecticide application records from 2005 to 2008.

Adults were collected during a 48 h-long sample period three times during the flight period in 2006, 2007, and 2008. In all years the first collection was made between 13 July and 19 July, the second between 27 July and Aug. 3, and the final one between Aug. 10 and Aug. 16. Beetles from both traps at each golf course were emptied into the same sealable plastic bag and placed in a cooler for transport to the laboratory where they were frozen. Within 6 mo of when the beetles were trapped they were removed from the freezer and thawed. Thawed beetles were sexed individually until 45–60 females were identified from each golf course for each sample period. Females were dissected to examine the ovaries and malpighian tubules. The number of developed eggs (>1.0 mm-long) in the ovaries was recorded for each female. Individuals were considered infected if the malpighian tubules contained sporophorous vesicles of *O. popilliae*, as previously described. Percent infection of females was calculated as the proportion of infected females to noninfected females for all three sample dates combined (a total of 136–180 females). The average number of fully formed eggs per female was also calculated for females collected on all three sample dates.

Statistical Analysis. Mean percent infection of Japanese beetle larvae with *O. popilliae* from holes on golf courses where *O. popilliae* was previously introduced was compared with the same from holes on nearby control golf courses, using the general linear models procedure (PROC GLM) of SAS 9.1 (SAS Institute 2002). Levene's test ($P \leq 0.05$) was used as part of the GLM procedure to test for *homogeneity of variance.* If variances were not homogenous, the nonparametric Kruskal-Wallis test was used (SAS Institute 2002). If variances were homogenous, means were separated at the $P \leq 0.05$ level using Tukey's option in the MEANS statement. The Tukey's Studentized Range test (honestly significant difference [HSD]) was performed when sample sizes of comparing groups were equal and the Tukey-Kramer test was used when sample sizes were unequal (SAS Institute 2002). The mean number of mature eggs per female from beetles collected on golf courses where imidacloprid was applied to all the fairways and all roughs (four golf courses) was compared with eggs per female on golf courses where imidacloprid was only used on some or all of the fairways but not on any of the roughs (six golf courses). Means were compared as previously described for infected larvae. The mean number of eggs per *uninfected* female at each golf course was compared with eggs per *infected* female at the same golf courses for all sites where a sample of 180 dissected females produced at least 12 females that were infected. A sample size of <12 was considered inadequate to estimate egg production per female. Means were compared as previously described for infected larvae.

Percent mortality from October to April at each site was calculated as 1 – (live larvae in April/live larvae

Table 3. Number of mature eggs found in Japanese beetle females on golf courses where imidacloprid is applied to all fairways and roughs compared with the same for golf courses where imidacloprid is applied to only the fairways or to only tees and putting greens

Treatment	Location	Number of females examined	Eggs per female	Treatment mean ± SD
Imidacloprid applied to all fairways and roughs each year	Bloomfield Hills CC	180	2.3	2.5 ± 0.2 A[b]
	Kalamazoo CC	162	2.7	
	Pine Lake CC	180	2.5	
	Orchard Lake CC	167	2.5	
Imidacloprid applied to fairways only, or to tees and greens only, each year[a]	Binder Park GC	136	5.2	4.8 ± 1.5 B
	Eastern Hills GC	155	2.8	
	Willow Metropark GC	159	4.7	
	Cracklewood GC	149	5.5	
	Medalist GC	170	6.8	
	Pine Valley GC	180	3.5	

[a] At Binder Park CC and Eastern Hills GC, the fairways only (no roughs) were treated with imidacloprid each year. At Willow Metropark and Cracklewood GC some of the fairways were treated each year but no roughs. At the Medalist GC only the tees and greens were treated with imidacloprid each year.

[b] Treatment means are not significantly different when followed by the same letter (Tukey-Kramer, Kruskal-Wallis, $P = 0.05$).

in October) × 100. To examine the relationship between *O. popilliae* infection of larvae and winter mortality, percent infection by *O. popilliae* was calculated for larvae collected at each site in October, and again for larvae collected at each site in April, then averaged. Percent infection was not calculated for a site unless at least 10 larvae were collected and examined for the presence of *O. popilliae.* The relationship of larval mortality from October to April to the mean percent infection of larvae with *O. popilliae* in October and April was determined by regression analysis using the SAS GLM Procedure with the model: grub mortality (y) = percent infection (x). The GLM Procedure of SAS was also used to determine if the incidence of detectable infection by *Paenibacillus* spp. was influenced by the proportion of larvae infected by *O. popilliae* at the same location (SAS Institute 2002). Percent infection data were converted to arcsine square root of % infection before analysis. Variance about means is reported as ±SD in all cases.

Results

Larval Sampling. Insecticide use on the 10 golf courses in southern Michigan where our research sites were located varied from golf courses that used insecticides only on tees and putting greens to golf courses that treated all of the tees, putting greens, fairways, and roughs, each year (Table 1). In all cases the superintendents were careful not to treat the turf areas in the irrigated rough where our sampling sites were located, even if the rest of that fairway was treated. Therefore, insecticides had no direct effect on survival of Japanese beetle larvae in our research plots or on the activity of *O. popilliae.*

Japanese beetle larvae infected with *O. popilliae* were found in October of 2000 at control sites in southwest Michigan where *O. popilliae* was introduced 5–15 km away in 1999 and 2000 (Tables 1 and 2). Apparently, *O. popilliae* had already been introduced into southwest Michigan before we introduced it in 1999 and 2000, because the incidence of *O. popilliae*-infected larvae was just as great at control sites as at introduction sites. In Southeast Michigan, however, *O. popilliae* was not found at control sites until 2006 and 2007, when 1.0–1.4% of the larvae were found to be infected compared with an infection rate of 20.3–20.8% at introduction sites (Table 2). Apparently, the introduction of *O. popilliae*-infected larvae in 1999 and 2000 resulted in the establishment of the pathogen at introduction sites in Southeast Michigan.

In 28 cases where the population of Japanese beetle larvae at sampling sites was great enough in the fall and following spring to make meaningful estimates of the population and percent infection, we found that winter mortality was greater at sites where *O. popilliae* was most active. At sites where <10% of the Japanese beetle larvae were infected with *O. popilliae*, mean mortality from October to April was 26% ($n = 12$; Fig. 2). When 10–30% of the larvae were infected, winter mortality increased to 43% ($n = 12$), and when >30% of the larvae were infected, winter mortality was 72%

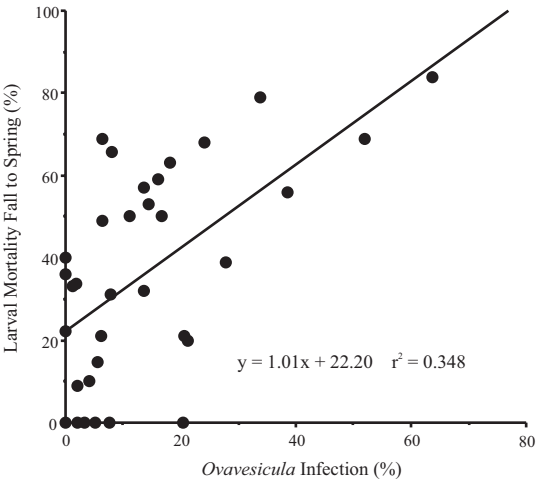

Fig. 2. Relationship of Japanese beetle larval mortality from October to April to mean percent infection by *O. popilliae* of larvae collected in October and in April at the same location.

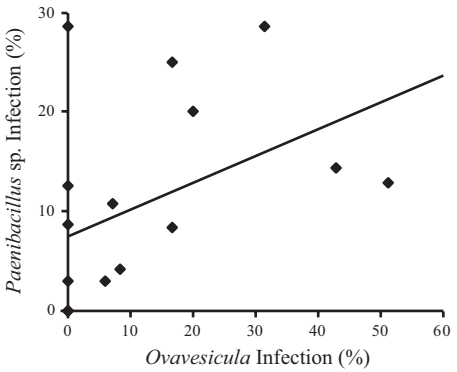

Fig. 3. Relationship of percent infection of Japanese beetle larvae by *Paenibacillus sp.* to percent infection of the same larvae by *O. popilliae* at 18 research sites in October 2005.

($n = 4$). Using regression analysis with the model: larval mortality = % infection (y = x), 35% of the variation in larval mortality can be explained by % infection of larvae by *O. popilliae* ($n = 28, F = 13.9, P < 0.001, r^2 = 0.35$; Fig. 2). Data from all 20 sites over a 3-yr period indicate a significant negative relationship between the incidence of *O. popilliae* infection of larvae and their winter survival (Fig. 2).

Although the milky disease bacterium, *Paenibacillus* spp., was detected in the hemolymph of larvae collected from 13 of 18 research sites in October of 2005, the number of *Paenibacillus* spp. sporangia tended to be low (<100 per 10 μl of hemolymph) in most cases, and general septicemia causing a milky appearance of the hemolymph was rare (<1%). Because of time limitations, October of 2005 was the only sample period when hemolymph was examined extensively for trace-levels of infection (10–100 sporangia per 10 μl of hemolymph). Regression analysis using this data from October 2005 indicates that the prevalence of *O. popilliae* in Japanese beetle larvae at 18 research sites was related to the proportion of larvae with a trace-level or low level (100–1000 sporangia per 10 μl) infection of *Paenibacillus* spp. When using the model % *Paenibacillus* spp. infection = % *O. popilliae* infection, the prevalence of *O. popilliae* explains 33.6% of the variation in the prevalence of detectable milky disease infection ($F = 8.1$, $P = 0.012$, $r^2 = 0.336$; Fig. 3).

Adult Sampling. Although no insecticide was applied to our research plot area, the intensity of insecticide use varied considerable among the 10 golf courses. Annual insecticide use from 2005 to 2008 at these sites fell into one of four insecticide use patterns: treatment of all of the fairways and all of the roughs ("wall to wall" or all of the turf on the golf course property, $n = 4$), treatment of all of the fairways, but not roughs ($n = 2$), treatment of some of the fairways ($n = 3$), treatment of tees and putting greens only ($n = 1$). When females of Japanese beetle ($n = 136–180$ per site) from each golf course were dissected and their ovaries examined, more mature eggs were found in the ovaries of females (4.8 ± 1.5, mean ± SD) from golf courses where only the greens, tees, and fairways were treated with insecticide compared with females (2.5 ± 0.2) from golf courses that treated all the fairways and roughs each year (Table 3). Statistical comparison of the means gave a significant general linear regression model ($\alpha = 0.05$; $F = 11.4$; $P < 0.01$) with a Tukey-Kramer minimal significant difference value of 1.9. Therefore, females collected from golf courses where all the fairways and roughs are treated annually with insecticide have significantly fewer mature eggs in their ovaries at any given time.

When dissecting females and examining their ovaries, their malpighian tubules were also checked for the presence of sporophorous vesicles of *O. popilliae.* The number of developed eggs in infected females was compared with that in uninfected females for five golf courses where at least 12 infected females were found (Table 4). The mean ± SD number of developed eggs per *uninfected* female at these five golf courses was 3.8 ± 1.6, compared with 1.9 ± 1.6 developed eggs per *infected* female. Tukey's HSD test (at $P \leq 0.05$) following general linear regression gave a minimum significant difference of 1.8 eggs per female (Table 4). Data from our sites indicate that only half as many developed eggs are found in infected females compared with healthy females.

Table 4. Number of mature eggs found in Japanese beetle females determined as uninfected or infected with *O. popilliae*, at five golf courses

Treatment	Location	Number of females examined	Eggs per female	Treatment means ± SD
Uninfected females	Binder Park GC	136	5.2	3.8 ± 1.6 A[a]
	Eastern Hills GC	155	2.8	
	Medalist GC	170	5.8	
	Orchard Lake CC	167	2.5	
	Kalamazoo CC	162	2.7	
O. popilliae-infected Females	Binder Park GC	20	2.0	1.9 ± 0.7 B
	Eastern Hills GC	25	3.0	
	Medalist GC	12	1.1	
	Orchard Lake CC	13	1.4	
	Kalamazoo CC	18	2.2	

Only five golf courses where at least 12 *O. popilliae*-infected females were found were used in the analysis.
[a] Treatment means followed by the same letter are not significantly different by a Kruskal-Wallis Test ($P = 0.05$).

Discussion

Returning to sites where *O. popilliae* was introduced 6–8 yr earlier, and to nearby control sites gave us a unique opportunity to study the establishment of *O. popilliae* (Table 1; Cappaert and Smitley 2002). In southwest Michigan, there was no difference in the incidence of *O. popilliae*-infection of larvae between introduction and control sites (Table 2). This may be because of the natural spread of *O. popilliae* from nearby sites where it had been found in 1999 before it was introduced into our research plots (Cappaert and Smitley 2002). In contrast, *O. popilliae* was not found at any of the 16 sites in southeast Michigan that were sampled in the same survey made in 1999 (Cappaert and Smitley 2002). In addition, *O. popilliae* was not found at any of the eight fairway sites on four golf courses in southeast Michigan until 2006, when the prevalence of infection was only 1.0%. Apparently, introduction of *O. popilliae* was successful in southeast Michigan because by 2006 infection levels averaged 20.3% where it was introduced compared with 1.0% at control sites (Table 2). Furthermore, in southeast Michigan *O. popilliae* spread from the 100 m^2 plots where it was introduced to plots surrounding a different golf course hole, 100–400 m away, over an 8-yr period. By 2006, 20% of all Japanese larvae found around both golf course holes near each introduction site were infected with *O. popilliae*, indicating successful establishment, build-up to an epizootic state, and spread of the pathogen at least 400 m.

Because two different holes at each golf course were used as replicates in the means separation test in Table 2, one can question if these are truly independent replicates. The additional hole at each course was added to the experimental design in 2005 when *O. popilliae* appeared to spread through control sites as quickly as introduction sites in southwest Michigan. Although two holes were sampled from each golf course, the means separation test was used to determine if golf course holes located near an introduction site have a mean infection level different from that at golf course holes not close to an introduction site. In southeast Michigan two additional golf courses (four additional replicates) were added to the control treatment (a total of four golf courses, eight holes) to make sure *O. popilliae* was not spreading from a natural introduction in the region, as appeared to happen in southwest Michigan. The absence of *O. popilliae* at all four control golf courses (eight holes) in southeast Michigan, and the presence of *O. popilliae* at all four control holes in southwest Michigan makes it unlikely that *O. popilliae* spread to the holes near our introduction sites in southeast Michigan from a previous introduction, although this possibility cannot be ruled-out entirely.

In southeast Michigan it is also possible that *O. popilliae* spread much further than 400 m from 1999 to 2006 because 1% of the larvae were infected with *O. popilliae* by 2006 at control golf courses located at least 5 km away from the nearest introduction site. The consistent recovery of infected larvae from turfgrass 400 m away from the introduction plot, and the recovery of infected adults at these sites, suggests that *O. popilliae* is also spreading from the movement of infected adults, because 400 m is too far to be explained by infected larvae moving in the soil. The likely spread of *O. popilliae* by infected adults is also supported by the fact that at sites where *O. popilliae* was active, 3–14% of the adults were found to contain sporophorous vesicles of *O. popilliae* with spores that are released into the soil and initiate infection after ingestion by larvae (Hanula 1990, Hanula and Adreadis 1992).

Because *O. popilliae* was found in southwest Michigan before it was introduced, it raises the question if the observed spread of *O. popilliae* in southeast Michigan could also have resulted from the natural spread of *O. popilliae* instead of spread from our introduction plots. Although this possibility cannot be entirely eliminated, it is highly unlikely that the four sites located within 400 m of an introduction plot averaged 20% infection by *O. popilliae* in 2006 while the eight sites located at least 5 km away from an introduction site averaged 1.2% infection in 2006 (Table 2).

Actual *O. popilliae* infection levels may be underestimated from two sample dates per year (October and April) because it is likely that some of the infected larvae died before the population was sampled. Our data indicate that the incidence *O. popilliae* infection is positively related to mortality rate but the actual cause of larval mortality from October to April is not known. We also did not attempt to look at mortality of larvae from August to October, or from April through June. Hanula (1990) did not observe any significant mortality of *O. popilliae*-infected larvae held in the laboratory when compared with healthy larvae. Our observations of the tissues of infected larvae support those of Hanula and Andreadis (1990); the infection tends to be localized in the malpighian tubules, where it causes extensive swelling and distortion. This may weaken larvae by impairing excretory function without causing rapid death of infected individuals. Further investigation is required to know how much of the mortality is caused directly by *O. popilliae* and how much is caused by an increased susceptibility to other pathogens as a result of the infection.

Data from October 2005 and April 2006 suggest that infection of Japanese beetle larvae by the milky disease bacterium, *Paenibacillus* spp. is more likely at sites where *O. popilliae* is active ($r^2 = 0.34$, $P = 0.01$; Fig. 3). Hanula (1990) also found that *Paenibacillus* spp. was epizootic at sites where *O. popilliae* was active, but suggested that the decline in populations of Japanese beetle was more related to a high rate of infection by *O. popilliae.* More research is needed to elucidate the interaction between *O. popilliae* and *Paenibacillus* spp., and to determine if infection by *O. popilliae* or other weak pathogens or parasites such as *Stictospora villani* Hays and Clopton (Eugregarinida: Actinocephalidae), may predispose larvae to infection and septicemia caused by *Paenibacillus* spp. (Hays et al. 2004).

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